THE UNIVERSITY OF ALBERTA

LIGHT AND ELECTRON MICROSCOPE STUDY OF THE ABORAL NERVOUS SYSTEM AND NEUROSECRETION IN THE CRINOID *FLOROMETRA SERRATISSIMA*

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Light and Electron Microscope Study of the Aboral Nervous System and Neurosecretion in the Crinoid Florometra serratissima" by Darcy Edward Goyette in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The aboral nervous system in the crinoid, Florometra serratissima has been examined for structural details and extent of its innervation, using both epoxy embedding methods for light and electron microscopy and paraffin embedding techniques. Functional morphology has been considered whenever possible.

The nervous system consists of a nerve ring near the base of the body, with nerve cords radiating out to innervate the cirri and surrounding arms. The appearance of the nervous system is one of a complex network of neuropile with neurons dispersed throughout the tissue. The nerve cells fall, typically, into three categories: a small bipolar neuron, comprising the largest population, and larger bi-, and multipolar neurons. The nerve cells are located in all areas of the nervous system, showing some tendency toward concentration near the cortical areas of the nerves, bases of the trunks leading to the arms and central regions of the nerve cords.

Many features commonly found in nervous tissue are also present in the aboral nerves. Neurotubules, neurofilaments, mitochondria and age-dependent lipofuscin pigment are commonly seen. Vesicles of two types can be found throughout the nervous system: large, membrane-limited vesicles (120-160 mμ) with dense contents, and clear vesicles (100-150 mμ). The former type are found in the cell bodies and along the nerve fibres, varying

in numbers depending on the individual. The latter, are typical of axo-axonic synapses and of neuromuscular junctions.

Innervation of the flexor muscles in each arm segment is accomplished through separate nerve tracts arising from the nerve cords. The nerves are restricted to the ends of the muscles and the anastomosing network of nerve fibres suggests a single innervation for each muscle cell. The myoneural junctions are characterized by the presence of synaptic vesicles and muscle mitochondria near the contact points.

Previous works have shown that the aboral nervous system is involved in the co-ordination and integration of many activities of the crinoid. These functions are reflected in the morphological appearance of the nervous system.

On the basis of morphology it is suggested that specialized neurosecretory neurons are present in the aboral nerves. This presumed neurosecretory activity is correlated with the occurrence of vesicles containing dense contents. Indirect evidence of cyclical activity in production of the vesicles in relation to reproduction suggests that crinoids may have a gamete shedding substance similar to that found in starfish.

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I. INTRODUCTION

Our understanding of the echinoderm nervous system is extremely limited, extending very little beyond the level of gross morphology and as clearly indicated by Smith (1965 and 1966) the analysis of nervous function has been restricted to a few preliminary and exploratory investigations. A great many problems remain unsolved and in order to achieve a closer understanding of the functional aspects of the nervous system and thus an insight into the biology of the echinoderms, further information is needed about the distribution of the nerves, myoneural relationships, and the ultrastructure of the nervous system. This will provide the basis on which to interpret new findings about the biological role of the nervous system in the echinoderms.

At the present time most of the attention has been directed towards the asteroids, particularly by Smith (1937, 1950, 1965, and 1966), with additional information on the myoneural physiology of echinoderms by Pople and Ewer (1954), Boltt and Ewer (1963) and on the ultrastructure of the hyponeural ganglia and lantern retractor muscle of the echinoid Echinus esculentus by Cobb and Laverack (1966a,b). The crinoids have received little attention beyond the account by Hamann (1889) who has provided the best general description of the nervous system in Antedon. The difficulty in obtaining specimens and the histological problems arising from their possession

of a calcified skeleton have contributed largely to this lack of attention. Since our understanding of the crinoid nervous system has not progressed much beyond that given by Hamann the purpose of this study has been to review his findings and provide additional information about the finer structure of the crinoid nervous system. With the advent of the recent methods for epoxy embedding for light microscopy and the greatly increased resolution provided by the electron microscope, much additional information is available to the observer and it is hoped that the observations presented in this study will lead to a better understanding of the role of the nervous system in the behaviour of the crinoid.

The crinoid has three more or less separate nervous systems, the ectoneural, the hyponeural, and the aboral nervous system. The aboral or so called "central nervous" system is the principle nervous system in the crinoid, controlling most of the major activities of the crinoid. The present study has been restricted entirely to this latter nervous system and will present a description of the structure of the aboral nervous system using light microscopy, supplemented for the first time by electron microscopy. During the course of this study several important aspects of the nervous system received special attention: the myoneural relationships and the evidence for neurosecretory activity in the aboral nervous system. Whenever possible in this account, correlations will be made between light and electron microscope observations and consideration given to the functional aspects of the various structures observed.

II. MATERIAL AND METHODS

A. Collection and Pre-treatment

Specimens of Florometra serratissima were collected in Satellite Channel, British Columbia, latitude 48° 41.9' N; longtitude 123° 28' W. Whenever possible collections were made by S. C. U. B. A. diving to insure a minimum of damage to the animals. The population lies just within the range of S. C. U. B. A. diving and the animals were taken from 15 - 18 fathoms.

When conditions did not permit diving the crinoids were dredged from 22 fathoms using the MV Hydah, Friday Harbor, Washington. Although many animals were damaged, enough could be collected in reasonable condition for the purposes of this study.

The crinoids were kept in fresh sea water aquaria at the Friday Harbor Laboratories for varying periods before fixation. Generally whole calyxes and arms were fixed for both light and electron microscopy. In some cases the nerve ring was isolated by microdissection before being embedded in order to remove as much calcareous material as possible. If the musculature lying around the calyx at the base of the arms was severed, the centrodorsal ossicle could be completely removed exposing the main portion of the nerve ring and primary nerve trunks. It was found that the outer portions of the nerve were usually damaged during dissection and only the interior tissue was fit for electron microscopy although the whole structure could be used for light microscopy.

B. Fixation for Light and Electron Microscopy

Tissue was fixed for 24 hours in Heidenhain's Susa (Pantin, 1962) for light microscopy and decalcification was completed in Bensley's decalcifier (Davenport, 1960) before final embedding. Following decalcification the tissue was dehydrated, cleared in xylene and embedded in paraplast (mp. 56-58°C).

For electron microscopy three different fixatives were used in this study. These were:

(1) 2% osmium tetroxide buffered with S-collidine (2, 4, 6 - trimethyl pyridine) at pH 7.48 (Bennett and Luft, 1959);

(2) 5% glutaraldehyde (Sabatani et al., 1963), in 0.27 M. NaCl buffered in Millonig's Phosphate buffer. Millonig's Phosphate buffer was made up at pH 7.4, 0.4 M. with respect to phosphate and 0.336 M. with respect to NaOH, following an unpublished procedure by P. Dunlap designed for ctenophores. The buffer was made as follows:

Sodium phosphate (monobasic) -- 11.04 gm.

10N. NaOH (40 gm. in 100 ml.) -- 6.7 ml.

Water to make -- 200 ml.

The phosphate was dissolved in 180 ml. water, then brought to pH 7.4. with NaOH and water was added

to make 200 ml. This fixative solution is hyperosmotic to Friday Harbor sea water and this hyperosmotic condition was maintained by rinsing in 0.4 M. phosphate buffer diluted with an equal volume of 0.6 M. NaCl. Post fixation was carried out in:

1 part 4% OsO_4

1 part 0.4 M. Phosphate buffer

2 parts 0.75 M. NaCl.

(3) 5% glutaraldehyde in Gomori's Buffer. (P. Dudley, unpublished). The fixative is hypersomotic to Friday Harbor sea water, rinse and postfixation is hypoosmotic. Fixative composition was:

1 part 5% glutaraldehyde in 0.27 M. NaCl.

1 part 0.4 M. Gomori phosphate buffer

Rinse was carried out in 0.2 M. Gomori buffer and postfixation was carried out in:

1 part 4% OsO_4

1 part 0.2 M. Gomori buffer at pH 7.4

Fixation time was 1 hour (1 1/2 hours for larger tissues) at 4°C. The glutaraldehyde was used in cases where the tissues were too thick for adequate fixation to be provided by osmium alone.

There were no obvious differences between the results obtained with the two glutaraldehyde fixatives and the osmium/S-collidine fixative with the

exception that microtubules were better preserved following fixation in glutaraldehyde.

C. Dehydration

After fixation and a brief wash in distilled water the tissue was dehydrated in the following graded series of ethanols: 30%, 50% and 70% ethanol for 10 minutes each, followed by two changes of 95% and 100% ethanol, each for 5 minutes. Dehydration was carried out at room temperature.

D. Embedding

Tissues for both light and electron microscopy were embedded in Epon 812 (Luft, 1961).

(1) Following two changes of propylene oxide for 10 minutes each the tissue was transferred to a 1:1 mixture of fresh propylene oxide and Epon and left overnight in an uncovered vial. Epon was made up of the following:

Epon 812 - DDSA (Dodecenyl-succinic anhydride) - 6 cc.

Epon 812 - NMA (Nadic Methyl Anhydride) - 4 cc.

Accelerator DMP-30 (2,4,6 - tri (dimethylaminanethyl phenol) -0.2 cc.

(2) After the propylene oxide had fully evaporated (12 - 15 hours) the tissue was transferred to a fresh mixture of Epon and polymerized for 20 - 24 hours at 60°C in No. 00 gelatine capsules.

E. Sectioning

Sections for both light and electron microscopy were cut with a Porter-Blum microtome (MT-2) varying at thicknesses from 600 - 1000 Å for the electron microscope, and lμ for the light microscope. Because of the presence of calcareous matter in the tissue surrounding the nerve, sectioning with glass knives is impractical. A diamond knife (DuPont) was used almost exclusively for cutting thin sections. Even so, sections were very difficult to obtain.

The thin sections were mounted on carbon coated 175 mesh grids for prolonged observations. Uncoated 300 mesh grids were used whenever good quality, unscored sections were available, and such preparations were chosen for more critical study and for photography.

The Philips EM 100B at 60KV using a 25μ objective aperature was used throughout this study.

Micrographs were taken on 35 mm Kodak fine grain positive film (P 426). Initial magnifications range from 3,500 to 7,000 and the pictures were further enlarged on printing. The magnification scale was

based on measurements taken from a grating replica having 28,800 lines per inch.

E. Staining

Epon Sections

For electron microscope studies the thin sections were stained with uranyl acetate and lead citrate. Grids were placed in 2% aqueous uranyl acetate and staining was carried out for 2 hours in the dark (Watson, 1958). Sections on grids were also stained in lead citrate (Venable and Coggeshall, 1965) for 1 minute followed by three thorough washes in distilled water. Uranyl acetate was found to provide a good general staining but lead citrate was necessary for the demonstration of neurotubules. The most satisfactory contrast was obtained by double staining for 2 hours in uranyl acetate followed by 1 minute in lead citrate.

For light microscope studies 1μ epon sections were cut and mounted on clean glass slides by drying on a hot plate. The sections were then oxidized in 1% periodic acid for 5 minutes followed by a 1 minute wash in running water. After allowing the slides to dry the sections were stained on the hot plate with a 1:1 mixture of 1% azure 2 in distilled water and 1% methylene blue in 1% borax (Richardson et al., 1960). The total staining period ranged from 15 - 30 seconds. Following a rinse the sections were dried and mounted in immersion oil.

Paraffin Sections

For routine observations 10μ sections of the calyx and arms were stained using Masson's trichrome method (Pantin, 1962). These gave the best results for general studies of the gross anatomy of the nerves. The special methods for silver impregnation of nerves by Holmes (see Carleton and Drury, 1957) and Peters (1955 a,b) and methylene blue vital staining were attempted but did not give satisfactory results. This has been a common experience among workers on echinoderms (Smith, 1965).

The method used to demonstrate neurosecretory material was the improved paraldehyde fuchsin staining method developed by Ewen (1962). With this method the stain was permanent and the stock solution would keep for one year without apparent change. Sections of the nervous system were stained following oxidation in acidified potassium permanganate as soon as possible after sectioning. Staining was carried out for approximately 30 seconds. To eliminate the possibility that a substance other than neurosecretory material was being stained, control slides of the same tissue were run concurrently omitting the oxidation procedure.

Tests for lipofuscin on the aboral nervous system were carried out using Sudan black B (Pearse, 1961; after McManus, 1946). Tissue used was fixed in Susa and embedded in paraffin.

Gomori's acetylthiocholine iodide was used to test for

cholinesterase activity. Frozen, free floating sections of the flexor muscle were cut and incubated according to the method given by Pearse (1961). Sites of cholinesterase activity could be demonstrated as shades of brown.

Tests for the presence of biogenic monoamines in the aboral nervous system were carried out on sections of freeze-dried isolated nerve rings using the fluorescence method for the cellular demonstration of biogenic monoamines outlined by Falck and Owman (1965). Six aboral nerve rings were isolated by microdissection and quenched in isopentane cooled to the temperature of liquid nitrogen. The tissue was stored in liquid nitrogen for two months before embedding. To prevent diffusion of the amines from their cellular sites during the treatment by paraformaldehyde the water content is very critical. Fresh paraformaldehyde and paraformaldehyde stored over sulphuric acid to give relative humidities of 50%, 70% and 95% were used to ensure optimal results (Hodgman et al., 1960). Microscopic analysis was carried out using Reichert binolux illumination with an Osram HBO 200 high pressure lamp as a source of UV-light. Schott BG 12 — filters were employed to give a wave length of 390-480 mμ. Controls were run simultaneously using rat adrenal medulla after perfection of the method using the stretch preparation of rat iris as outlined by Falck and Owman (1965). The tissue generally exhibited a prominent green autofluorescence and to show that this was not due to monoamines, a sodium borohydride reduction was carried out as a specificity test (Corrodi et al., 1964).

III. OBSERVATIONS

1. GENERAL DESCRIPTION OF THE ABORAL NERVOUS SYSTEM IN FLOROMETRA

The overall pattern of the aboral nervous system in Florometra is to a large extent similar to that described for other crinoids, particularly Antedon mediterranea described by Hamann (1889) and reviewed by Hyman (1955). Yet it is hoped that the following account of the gross morphology of the aboral nervous system which is based on light microscope observations will serve to familiarize the reader with crinoid morphology and will provide the foundation for the more detailed account of the light and electron microscope observations that will follow. Whenever possible descriptions have been based on tissues embedded in Epon, since the preservation of the cellular details in this material is far superior to that of tissues embedded in paraffin.

The aboral nerve ring in Florometra lies within the cavity of the centrodorsal ossicle, a series of radial plates forming the major part of the comatulid calyx. It is centrally located, appearing as a cup-shaped mass of nervous tissue surrounding the aboral extension of the body cavity, the chambered organ (Figs. 1 and 2).

From the dorsal portion of the nerve ring numberous radial extensions of the chambered organ pass dorsally out through the nerve

ring to the adjacent cirri and from there they continue throughout the whole length of the cirrus. Enveloping each tubular extension of the chambered organ is a sheath of nerve fibres passing out from the aboral nerve ring, serving as a principal nerve supply to the cirri.

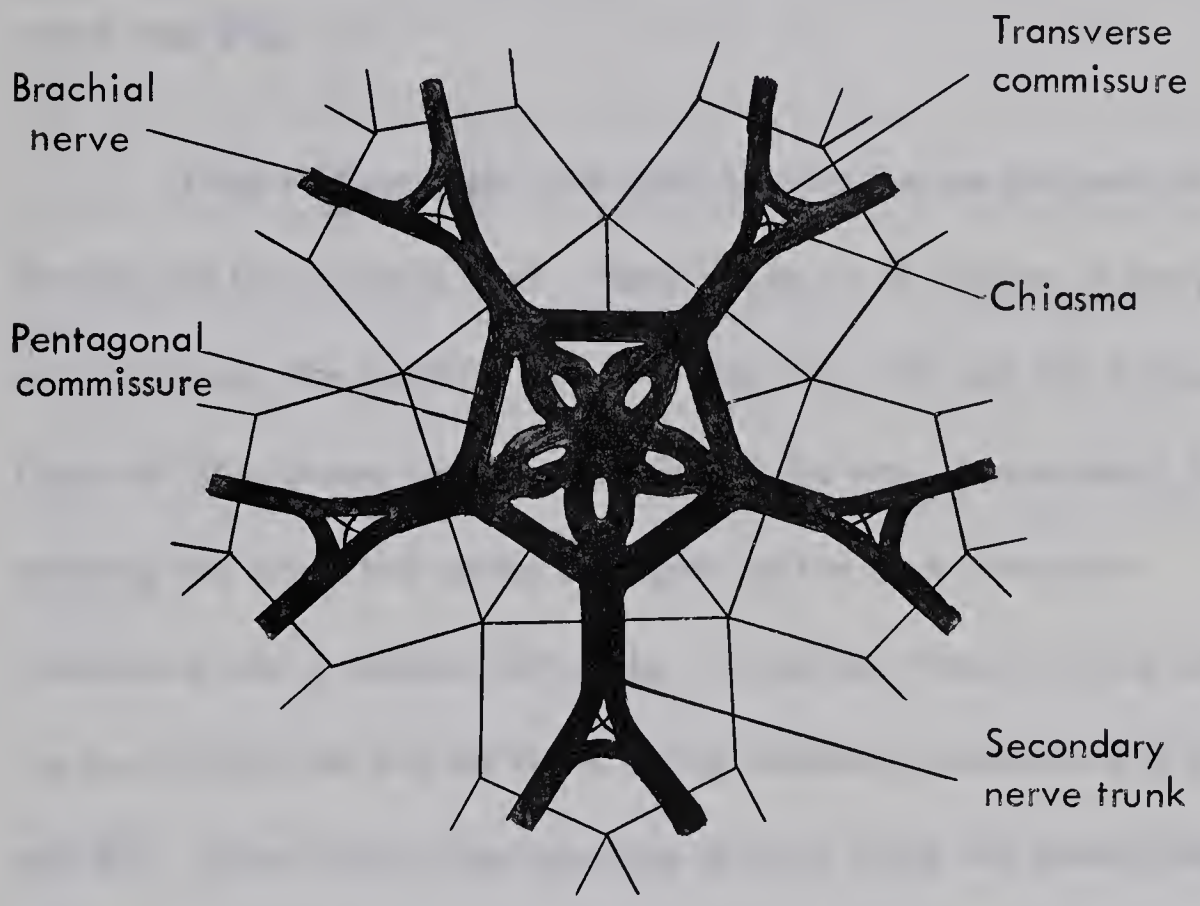


Fig. 1. A diagram of the aboral nerve ring (after Hamann, 1889).

Immediately above, five large primary nerve trunks pass diagonally from the main nerve mass, where they continue orally, soon to divide into ten. The two halves resulting from the division of each primary trunk diverge and unite with the similarly diverging halves of the two adjacent nerve trunks to form the secondary nerve

stems (Fig. 1). These secondary nerve stems are located within the radials (five plates, one at the base of each pair of arms) and will eventually form the axial cords or brachial nerves of the arms. Shortly after the formation of the secondary nerve stems the five trunks are again united by a pentagonal commissure concentric with the main nerve mass (Fig. 1).

From this pentagon five main brachial nerves proceed out through the five primary arms. Near the point of division of the primary arms into two, the brachial nerves divide into right and left halves. Each half then passes into one division of the arm. Immediately before entering the arms, the nerves are again united by a transverse commissure and a chiasma formed by two oblique fibres running between the nerve branches and the bases of the transverse commissure (Figs. 1 and 29). These nerves then continue distally along the dorsal portion of each arm as the brachial nerve cords. In each segment of the arms the nerve cords send branches to the flexor muscles and adjoining epidermis, pinnules and aboral surface of the arms.

2. HISTOLOGY OF THE ABORAL NERVOUS SYSTEM

A. Aboral Nerve Ring

i. Light Microscope Observations

In paraffin sections the fibres of the aboral nerve ring in the region of the chambered organ appear to run concentrically around the

chambered organ, with the cell bodies scattered throughout the tissue. The ring is interrupted periodically by entrance of the cirral nerves and the fibres of the five primary trunks that pass ventrally as parallel fibres.

In epon sections this concentric pattern is not shown. Instead, a complex mass of interwoven nerve fibres is seen. It would appear that the fibres although they course concentrically about the chambered organ, probably to provide for complete communication between the five primary trunks and cirral nerves, travel in a rather tortuous path about the nerve ring. Thus, in a 1μ section, the circular orientation would not be apparent.

There is little evidence of a sheath or neurolemma surrounding the nerve ring and for the most part the nerve ring can be considered contiguous with the surround calcified connective tissue (Fig. 2). However, in certain areas a diffuse network of elongated cells ("neurolemma cells") containing deeply staining oval structures approximately 1 to 2 microns long does occur along the nerve connective tissue interface. In Antedon the condition appears to be fairly similar to that found in Florometra. Similar cells containing oval structures have been described by Hamann and Reichensperger (cited by Clark, 1921). These cells are diffusely distributed around the nerve ring or in many areas absent. They have, nevertheless, been held to play an important

role in regeneration (Clark, 1921). In Tropiometra carinata by contrast, Hamann (cited by Clark, 1921) describes a very thick sheath surrounding the cirral nerves and aboral nerve ring. The capacity for regeneration is likely to be better developed in Tropiometra than Antedon since the former is more fragile and subject to constant breakage. This could possibly explain the presence of the more highly developed sheath around the nerves in Tropiometra.

In the nervous system of Florometra the cell bodies of the neurons are distributed throughout the nerve ring, showing some tendency towards concentration near the cortex of the nerve ring and bases of the nerve trunks leading to the arms, but never being present in sufficient numbers to form discrete ganglia. The cell bodies lie surrounded by the neuropile formed from the nerve cell processes (Fig. 3). From studies of the cell bodies three basic cell types are recognizable in the aboral nerve ring. The first is a small bipolar type, characterized by a darkly staining nucleus approximately 3.0 to 4.0μ in diameter with the chromatin occurring in clumps. The cytoplasm stains lightly and it is consequently hard to distinguish the shape of the perikaryon. These small bipolar cells comprise the largest population of cells present and are distributed randomly throughout the entire nerve ring. The remaining two cell types are the relatively large bipolar and multipolar ganglion cells (Fig. 3). Both show a large

nucleus approximately 8μ in diameter, containing finely dispersed chromatin and a prominent nucleolus. The perikaryon is very conspicusous in both size and in the stainability of the cytoplasm, which generally contains numerous granules. These granules vary in size from extremely fine particles up to .5μ in diameter and in some cases can be traced for some distance out along the processes of the cells (Fig. 3). There is evidence to show that these large ganglion cells may function in neurosecretion. The significance of this will be apparent in the following section dealing with neurosecretion. Although difficult to measure precisely, the cell bodies of both types not including their processes range from 15 to 20μ in length. These larger, less numerous cells can be found throughout the entire nerve ring but do appear to be concentrated in certain areas of the nerve. Heavier concentrations are generally found in the cortical areas of the nerve ring and at the bases of the primary and secondary nerve trunks. Their processes extend out in all directions to mingle with the processes of adjacent nerve elements. Similar bipolar and multipolar ganglion cells with comparable distribution have been described in other crinoids studied (Clark, 1921) but no mention of their possible neurosecretory activity has yet been made.

In many of the animals studied the nerve ring contained large quantities of yellowish-brown pigment granules so far not described in any accounts on the crinoid nervous system. This pigment appeared as

several irregular shaped granules of approximately 1.0μ in diameter seen within the nerve cell bodies, up to large clusters ranging around 20 to 25μ in diameter (Fig. 4). This pigment can be observed in fresh preparations of the aboral nerve ring, to which it gives a speckled appearance and heavier concentrations of this pigment appear to represent areas of concentrations of the nerve cell bodies, for instance in the cortex of the nerve ring and at the bases of the nerve trunks (Fig. 5). The pigment is not restricted entirely to the nervous system, but can be found out in the surrounding tissues. It is insoluble in the reagents used in paraffin and epon embedding and tests with Sudan black B suggest, with a high degree of probability, that these pigment granules have a high lipid content. The amount of pigment present is dependent upon the age of the individual. It appears to be almost totally absent in young animals observed in both fresh and sectioned condition, and increases to extremely heavy concentrations in the older animals (Fig. 4). The above mentioned properties of this pigment suggest that it is a lipofuscin, comparable to that found in the nervous system of many organisms. It is considered by many to be a so-called "wear and tear" pigment consisting of insoluble residues left behind after the breakdown and digestion of the cell organelles. In most organisms including the crinoid it can be found in large quantities in older animals and in many cases it is found in degenerating nerve cells.

It is necessary to distinguish the lipofuscin pigment found in nerve cells from pigment masses found in certain non-nervous cells scattered throughout the nervous system. These cells may appear singly or in clusters of up to eight. They resemble the "neurolemma cells" mentioned above (page 14). The pigment consists of oval granules 1 - 2μ in length, aggregated in masses (Fig. 6). The cytoplasm of these cells, whose function is as yet unkown, shows a yellow primary fluorescence under UV-light.

The aboral nerve is associated with certain non-nervous tissues, which may be briefly discussed at his point. In Florometra the chambered organ is divided into six separate chambers by radiating septa (Fig. 2). Each individual chamber is lined with an endothelial lining varying from a single cell layer approximately 4 to 5μ thick in the region of the cirral nerve (Fig. 7) to a layer several cells deep and approximately 12.0 to 13.0μ thick near the bases of the primary trunks. At the entrance of the cirral nerves the endothelial cells are slightly larger, forming the lining of the cirral canals. Approaching the region of the primary nerve stem, the lining of the chambered organ becomes progressively thicker and exhibits several cell types (Fig. 8). The majority of cells (Fig. 8, a) in this area contain deeply staining nuclei in which the chromatin occurs in clumps. The nuclei measure approximately 4 to 5μ in diameter and generally exhibit a variety of

shapes. The second type (Fig. 8, b) has a large oval or round nucleus approximately 6μ in diameter usually with a prominent nucleolus and relatively little chromatin. Interspersed within this tissue are numerous large vacuoles some appearing to be empty while others contain a substance showing high metachromasia with the azure 2 of Richardson's stain. These cells continue out along both sides of the septa making up the major bulk of the septa. Further in toward the centre of the chambered organ, the septal cells become highly vacuolated and appear to be secreting a similar metachromatic substance to that seen coming from the endothelial cells (Figs. 9, 10, and 11).

The cavities of the chambered organ contain numerous spheres of 3 to 4μ in diameter and large masses of finely granulated network of a similar metachromatic substance. It appears that the cells lining the chambered organ and septa are actively secreting this substance found within the cavities (Figs. 9 and 10). Similar such speres measuring 6 - 10μ have been described in other species of crinoids studied by Perrier and Reichensperger (cited by Clark, 1921). Reichensperger suggests that the substance comprising the fine granular network and the spheres may be formed by the endothelium of the chambered organ and plays some role in calcification. He could not, however, determine the origin of the

substance. He further noted that in specimens of Antedon where regeneration of whole arms was taking place very few spheres were present, but instead a network of strongly stained substance resembling that enveloping these spheres was present in the chambered organ. Sections taken of Florometra do in fact indicate that the substance is coming from the endothelial cells. The high metachromasia, although it cannot be regarded as providing histochemical proof, does suggest the presence of a highly sulphated mucopolysaccharide. Since mucopolysaccharides are capable of chelating divalent metals, it would suggest that the substance within the chambered organ is responsible for the chelation and storage of calcium salts for use in the repair and growth of the skeleton.

ii. Electron Microscope Observations

The Neuropile

The neuropile of the aboral nerve ring consists of a tangled network of many hundreds of unmyelinated nerve fibres (Fig. 12). Each fibre appears to trace a tortuous path about its neighbours, giving the sections a similar appearance regardless of the plane of the sections. The fibres can vary considerably in diameter but the majority range around 1 - 2μ. Numerous lateral branches coming from the fibres are a common feature in the aboral nerve (Figs. 13 and 14). They can extend for considerable distances and usually have periodic

swellings along their length. In most cases these swellings contain vesicles and/or mitochondria and at times almost appear to communicate directly between two nerve fibres (Fig. 14).

Within the nerve fibres various types of structures can be discerned. Common in glutaraldehyde-fixed tissue are neurotubules (Figs. 15 and 16) measuring approximately 280 Å in diameter. Not all the nerve fibres contain neurotubules. It has not been determined whether or not they are restricted to either the axon or the dendrites. Also present along the nerve fibres are fine networks of neurofilaments, a few mitochondria and at times profiles of smooth endoplasmic reticulum are seen.

A prominent feature of the nerve fibres are vesicles, of which two types are most common (Fig. 17). Occurring consistently in all animals are large clear vesicles ranging from 1000 Å to 1500 Å in diameter. They can be found throughout the aboral nerve either scattered singly or in heavy concentrations along the nerve fibres as shown in Figure 17.

A second major type of vesicle (referred to here as "dense core vesicle" also shown Fig. 17) is one containing a dark osmiophilic centre separated from its surrounding membrane by an electron lucid periphery. These vesicles range between 1200 Å and 1600 Å in

diameter with the dense core varying from a small granule in the centre of the vesicle to a mass so large that it is tightly juxtaposed against the limiting membrane, obscuring the latter. This gives the impression of a third type of vesicle but indications are that it probably represents a stage in the synthesis of the substance within the vesicle. In a recent study on the fine structure of the hyponeural ganglia of Echinus esculentus similar vesicles were indicated (Cobb and Laverack, 1966a). The diameters vary slightly from those of Florometra with the clear vesicles ranging around 40mμ and the dense core vesicles from 70 to 100mμ.

So far in the study of the aboral nerve areas of synaptic contact have not been readily demonstrated. Figure 18 shows what is presumed to be a synaptic region representing a two-way type of axo-axonic synapse. Clear vesicles are present on both the pre- and post-synaptic sides of the membrane. The membranes near the presumed synapse are quite diffuse and there is no apparent synaptic cleft. Similar presumed axo-axonic synapses have been shown to be present in the echinoid Echinus esculentus (Cobb and Laverack, 1966a). They have demonstrated that twenty such contact areas can be seen at once within the hyponeural ganglia. Most of these areas also exhibit a diffuseness in the membranes near the area of contact and show no apparent synaptic cleft. It was also indicated that synaptic areas

are restricted to certain portions of the hyponeural nervous system. A similar localization of synaptic areas may occur in the aboral nervous system of Florometra since evidence of synaptic contact has been extremely rare even where fibres come in close contact with the nerve cell bodies (Fig. 12) and it is possible that none of the areas examined in this study happened to include synaptic areas.

There is no evidence of any glial or satellite cells in the aboral nerve in Florometra. The only indication of non-nervous cellular elements is the occasional occurrence of the cells containing the oval structures mentioned previously (page 14). Figure 19 shows the typical fine structures of these cells. They lack the common features of the nerve cell, the cytoplasm being almost entirely filled with these oval packets. The size of the oval packets is approximately 2.0μ x 1.0μ.

The chief means of support in the aboral nerve appears to be strands of collagen fibres that can frequently be seen running throughout the nerve (Fig. 20). Specific supportive cells found in the radial nerves of asteroids (Smith, 1937) seem to be absent in the crinoid. It has been noted that fresh nerve tissue was extremely delicate and easily torn in dissection, suggesting that there is

little supportive tissue within the nerve. Since the nervous system in the crinoid is completely surrounded by highly calcified connective tissue there appears to be little need for any support within the nerve.

Components of the Nerve Cell Bodies

The cell bodies interspersed among the nerve fibres generally contain many of the features common to nerve cells of other animal groups (Fig. 21) (see Bullock and Horridge, 1965).

The nuclei exhibit a variety of shapes, their chromatin contents being either in clumps or in a fine network dispersed evenly throughout the nucleus. The nuclei exhibiting the finely dispersed chromatin probably correspond to those of the large bipolar and multipolar ganglion cells seen by light microscopy. A large nucleolus is usually associated with similar nuclei. Surrounding the nucleus there is a prominent nuclear envelope (Fig. 21).

Mitochondria are always present throughout the cytoplasm (Fig. 21) and they are generally round or oval, approximately .6μ long and .4μ wide. The cristae are few in number and rather difficult to distinguish.

Rough endoplasmic reticulum is a conspicuous feature in the nerve cell body (Fig. 21). It can be found throughout the cytoplasm as a series of double membranes lined with numerous

ribosomes. At times it is represented by a circular or eliptical profiles (Fig. 21). In some cases the endoplasmic reticulum appears to be continuous with the nuclear envelope (Arrow). The amount of endoplasmic reticulum present in the nerve cells is highly variable and appears to correspond to the concentration of the dense core vesicles present in the nerves. Large quantities of dense core vesicles are usually accompanied by an increase in the amount of endoplasmic reticulum present. Free ribosomes can also be found throughout the cytoplasm either singly or most commonly as tiny rosettes (polyribosomes). Smooth endoplasmic reticulum so far has not been found to be a common feature in the cell bodies. The presence of rough endoplasmic reticulum, numerous ribosomes and a prominent nucleolus suggests that the nerve cells are actively involved in protein synthesis.

Figure 21 shows a well developed Golgi complex as a series of concentric membranes and numerous Golgi vesicles associated with them. These vesicles vary in size from 900 $\overset{\circ}{A}$ to 1500 $\overset{\circ}{A}$ in diameter and appear as circular clear vesicles. In most cases the Golgi complexes are smaller and not as well developed (Fig. 22). The Golgi complex in Figure 22 is the more common type found, appearing as a series of flattened parallel membranes.

Numerous membrane-bounded bodies of unkown nature have been found in the nerve cells of Floremetra (Fig. 21). Contained within these structures are numerous small vesicles and segments of membranes. It has been suggested that these multivesicular bodies may be pre-lysosomes (Smith and Farquar, 1966): an alternative possibility is that they play a role in the formation of neurosecretory products, representing an intermediate stage in the transformation of neurosecretory material into larger droplets (Palay, 1960).

Associated with many of the nerve cells are structures comprised of a series of lamellated smooth membranes (Figs. 22, 23, 24, and 25). They occur in a variety of forms ranging from several concentric membranes approximately 0.23μ in diameter up to numerous coiled membranes bounded by an additional series of outer membranes (Fig. 24). The size of these lamellate bodies can range up to 2.0μ in diameter. In many cases these lamellate bodies contain a highly osmiophilic substance between the membranes (Figs. 22 and 25) and visual evidence suggests that this substance is being actively formed within the membranes, progressively increasing to the point where the membraneous structure of the lamellate body is totally obscured, resulting in the final formation of large granules ranging around 1.0 - 2.0μ in diameter (Figs. 25 and 26). Comparison of these granules with similar granules seen

by light microscopy reveals that they correspond to the sundanophilic granules described previously to be a form of lipofuscin pigment.

These lamellate bodies appear to be a type of lysosome or preferably "a primary lysosome" which is actively involved in the formation of the presumed lipofuscin pigment. They begin as a series of lamellated smooth membranes which progressively accumulate a lipofuscin pigment into larger granules. So far these granules have only been found in the cell body, never in the cell processes. Similar sudanophilic granules have been described in neurosecretory cells of gastropods (Lane, 1966) and probably correspond to the lipochondria found in many invertebrates and similar lamellate bodies have been described by Palay and Palade (1955).

Electron micrographs of older animals containing the large masses of pigment previously described in the light microscope observations show that these masses consist essentially of heavy concentrations of similar granules (Fig. 27 and 28) to the extent where the cytoplasm of the cell is almost totally obscured (Fig. 28). The cells containing the granules have numerous highly lobulated nuclei suggesting that they are undergoing degeneration. It would appear that these large

masses are formed from the accumulation of numerous lipofuscin granules which originate from a type of lysosome.

The ultimate fate of this pigment has not been determined but in some cases spaces can be seen around the granules as if to indicate a progressive removal of the pigment from the cell (Fig. 28). Lipofuscin has been previously described in the echinoderms by Millott and Vevers (unpublished) who have demonstrated its occurrence in the axial gland of certain echinoids and by Dimelow for the crinoid Antedon bifida (cited by Boolpotian, 1966).

B. Brachial Nerve

i. Light Microscope Observations

As indicated previously the secondary nerve trunks pass out the dorsal portion of the base of the arm as the brachial nerve. Prior to the division of each arm from its common base the brachial nerve undergoes a similar division to be again united by a transverse commissure and a chiasma. Figures 1 and 29 shows this peculiar arrangement of communicating fibres located in the axillary brachials of the arm. The fibres in the transverse commissure mesh with the fibres of the adjacent right and left branches. Numerous cell bodies can be seen along the commissure

particularly near the bases. The two oblique fibres comprising the chiasma consist chiefly of parallel fibres joining the opposite halves of the nerve branches. The fibres of each oblique strand remain separate from those of the other where they cross in the centre and the strands of the chiasma are almost entirely devoid of cell bodies. This distinct network of communicating fibres suggests that the brachial nerve contains separate nerve tracts. From physiological experiments conducted by Marshall (1884) and Moore (1924) on Antedon rosaceus it seems probable that the brachial nerve cords and the aboral nerve ring contain both afferent and motor fibres. This arrangement could allow in some cases for the co-ordinated action of two arms having a common root, without mediation of the aboral nerve ring. In other cases if a stimulus is of sufficient strength the remaining arms can be affected via the aboral nerve ring. Preliminary tests carried out in this study on Florometra show that if a stimulus of low strength is given to a single arm the response is confined to that arm and its partner of the common root. If, however, a stronger stimulus is given there is a response in the remaining arms, the response appearing first in the arms nearest the ones showing the initial response, and appearing in the two opposite arm-pairs only with a very strong stimulus.

Beyond the transverse commissure the brachial nerve passes along the dorsal portion of each arm as a solid cord approximately 100μ in diameter near the base and diminishing in size as it passes distally. It lies completely encased within the calcified tissue of the brachials (Fig. 30).

Small bipolar and larger bipolar and multipolar ganglion cells similar to those described in the aboral nerve ring can be seen along the length of the brachial nerves (Fig. 31). The large ganglion cells chiefly occupy the cortical areas of the nerve cord and at times cell bodies lie out in the surrounding connective tissue. In addition to this further concentrations can be seen in the central areas of the nerve. In the central portion of the nerve cord cell bodies occur in irregular chains lying around a central strand of larger nerve fibres (Fig. 31). Between the outer rind of cell bodies and the central chain of cell bodies is the neuropile. The significance of this arrangement of cell bodies and nerve fibres has not been determined but it seems to fit in with the concept of the nerve cord as a system in which there are discrete integrative areas and also separate nerve tracts possibly representing afferent and efferent pathways. The central areas of the nerve cords would appear to consist of larger parallel nerve fibres en route to distant points, surrounded by cell bodies possibly linked in series and connected with some of these parallel fibres. The neuropile surrounding

this central core likely contains the bulk of the cell processes and is involved in the integrative events occurring in the nerve cords. This spatial subdivision of the nerve cords into discrete areas can only be observed in epon sections.

Sections of the nervous system have shown that the tract of exceptionally large fibres passes along the centre of the nerve cord down to the aboral nerve ring where it continues along the outer edge of the aboral nerve (Fig. 32) to join with a similar tract coming from the adjacent arms. In the region of the chiasma this fibre tract appears to be restricted to the main portion of the nerve branches and does not pass out along the chiasma or transverse commissure. These large fibres range from 3 - 6μ in diameter.

Lipofuscin pigment is also present in the nerve cords in the form of numerous granules up to approximately 1 - 2μ in diameter within the cell bodies (Fig. 33), and often occurring in large clusters (20μ) similar to those found in the aboral nerve.

The neurolemma surrounding the nerve cords remains similar to that found about the aboral nerve ring consisting again of a diffuse network of elongated cells containing oval granules of approximately 2.0μ in length.

ii. Electron Microscope Observations

The fine structure of the brachial nerve is essentially similar to that of the aboral nerve, consisting of a tangled network of unmyelinated nerve fibres with numerous cell bodies interspersed among the fibres. Neurotubules, neurofilaments, mitochondria and vesicles both with and without dense contents can be found within the nerve fibres. Figures 34 is a section taken from the region of the large fibre track found in the centre of the brachial nerve. The fibres range up to 5.0μ in diameter, noticeably greater than the small fibres also shown (Fig. 34). The interior of the fibres is relatively devoid of structure, but a network of neurofilaments, several vesicles of both types and a few mitochondria are seen.

C. Branches of the Brachial Nerve

In each brachial segment of the arm the nerve cord repeatedly gives off lateral branches which supply the various parts of the arms (Figs. 35 and 36). Near the brachial ossicle two stout branches pass dorsally, penetrate the calcified tissue of the ossicle and extend to the dorsal epithelium (Fig. 35). Additional branches also pass to the dorsal ligaments, ending at a group of deep-staining cells located in a bow-shaped configuration near the point of attachment of the ligaments to the brachial ossicle (Fig. 36). These cells are pear shaped and contain a highly vacuolated cytoplasm, probably indicative of large amounts of endoplasmic reticulum. The nucleus usually contains a prominent nucleolus.

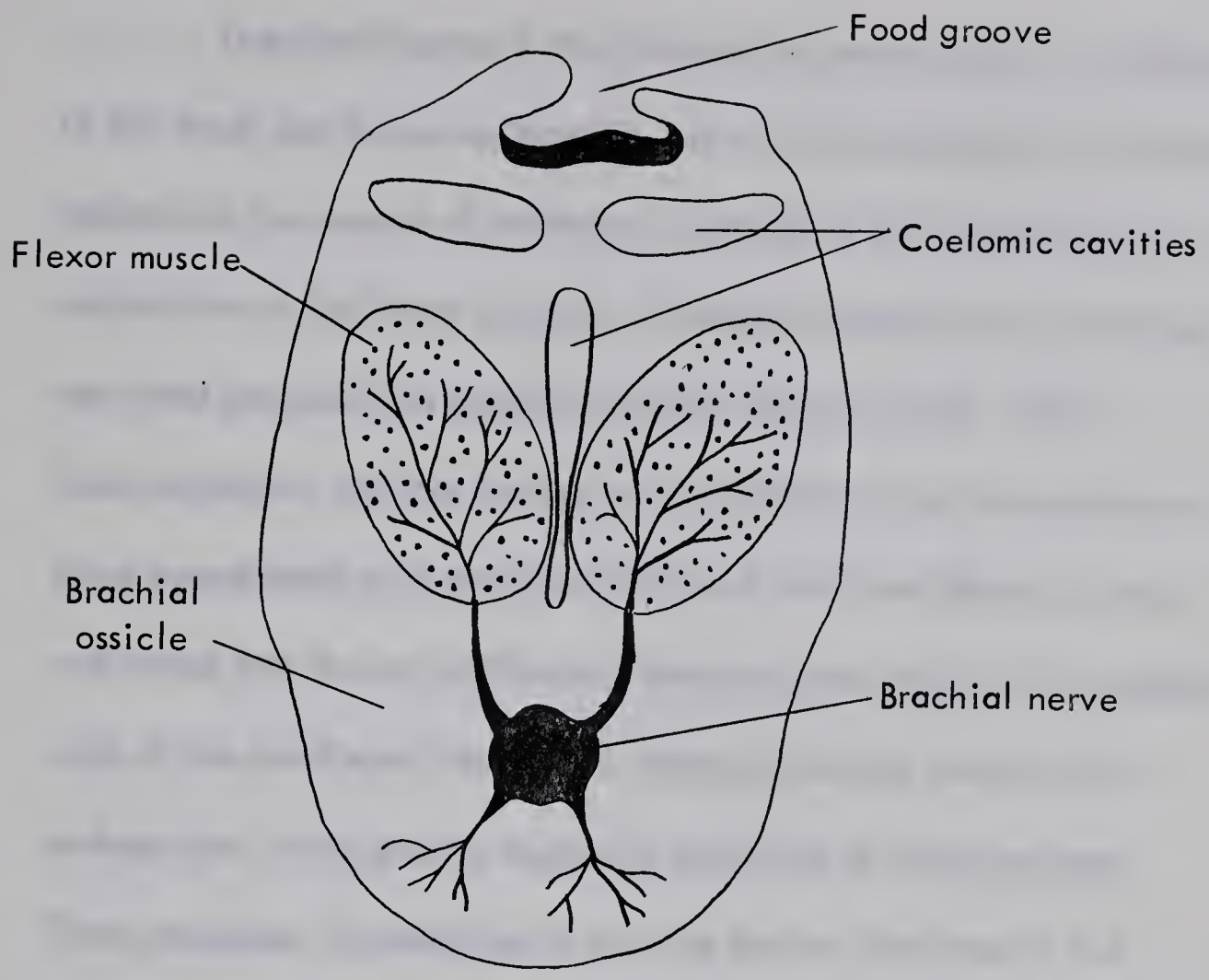


Fig. 35. Diagram of a cross section of the arm showing the branches of the brachial nerve going to the flexor muscles and the brachial ossicle. Also see Fig. 30.

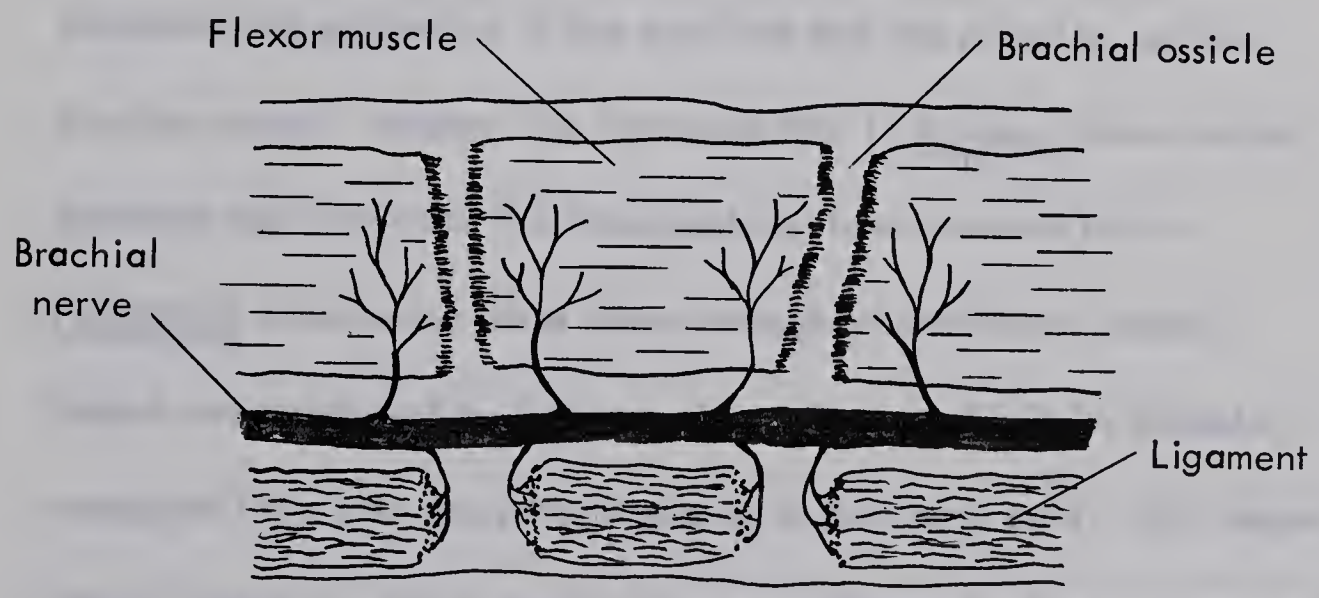


Fig. 36. Diagram of a longitudinal section of the arm showing the branches of the brachial nerve going to the flexor muscles and the dorsal ligaments. Also see Fig. 37.

The significance of this innervation has not been investigated in this study but the nerves possibly have some proprioceptive function, registering the amount of extension occurring in the ligaments during contraction of the flexor muscles. These pear-shaped cells have been described previously as ganglion cells by Perrier (Clark, 1921). Reichensperger, who has worked out the details of the connections of these pear-shaped cells and the axial cord confirmed Perrier's views and stated that the only difference between these cells and the ganglion cells of the axial cord lies in their stronger staining reaction and perhaps also in the greater degree of branching of their processes. These processes, he describes as running betwen the fibres of the ligaments.

Two branches pass from the nerve cord in the ossicle to innervate the epithelium of the oral side and the pinnules, as the pinnule nerves. Hamann has indicated that in Antedon these ventral branches also innervate the interbrachial flexor muscles but in Florometra innervation takes place through an additional lateral branch not mentioned by Hamann, approximately 12.0μ in diameter, somewhat finer than the other branches of the nerve cord. This lateral branch leaves the nerve cord in the area adjacent to the attachment of the flexor muscle to the brachial ossicle (Figs. 35, 36, and 37). At this point the nerve fibres can be traced to the muscle bundles where,

upon entering the muscle fibres, the nerve splits into further subdivisions which traverse the entire width of each flexor muscle. The nerve fibres become finer and more difficult to trace as they pass across the muscle, sending numerous lateral branches that anastomose and ramify throughout the muscle fibres (Fig. 38). Small bipolar nerve cells can be seen along the length of the nerve as it passes through the muscle. With the extreme fineness of the terminal branches of the nerve in the muscle bundles, it is difficult to resolve the exact mode of innervation. It does, however, appear that only one or two nerve fibres are present at the points of innervation. Smith (1950) describes in the starfish Astropecten irregularis, structures called "ribbon axons" – large axons which overlie the muscle fibres of the ampulla. No sign of such objects was seen in the present material, even with electron microscopy. Such figures as (Fig. 38) would be expected to show such structures if present. Cobb and Laverack (1966b) have also failed to find evidence of ribbon axons in the echinoids. Innervation is restricted to the regions near the attachment of the flexor muscle to the brachial ossicle and nerve fibres have not been found in the central region of the muscles. Lateral branches of the nerve pass to each end of a single flexor muscle. Hamann (1889) has described the flexor muscle innervation to its entrance into the muscle bundles but beyond that point the relationship of the nerve to muscle has not been described.

D. Muscle Structure

Papers by Kawaguti (1964a, b) and Kawaguti and Kamishima (1965) have described the fine structure of the muscles in holothurians and echinoids, respectively and Cobb and Laverack (1966b) have presented the fine structure and innervation of the lantern retractor muscle of the echinoid. Beyond this little is known about the muscle structure and the neuromuscular junctions in the echinoderms.

The flexor muscles located in each segment of the arms of Florometra are composed of numerous cylindrical bundles, each in turn is composed of numerous wedge-shaped striated fibres with their apices pointing to the centre of the muscle bundle (Fig. 38). In the paraffin sections the actual arrangement of the fibres is somewhat distorted from the treatment. The fibres are separated in paraffin sections but this made it possible to trace the nerve fibres within the muscle. Each flexor muscle is attached at its two ends to a brachial ossicle by a series of finger-like projections extending out from the muscle into the ossicle (Fig. 37). Each muscle fibre is approximately 3μ in diameter with numerous small nuclei approximately 6μ long lying along the periphery of the fibre. The ultrastructure of the flexor muscle is shown in Figure 39. Numerous mitochondria can be seen throughout the muscle. The cristae are well developed and extend well into the lumen of the mitochondrion. Associated with the nuclei of the muscle

cell are large amounts of sarcoplasmic reticulum and numerous droplets resembling what some workers describe as triglyceride. Additional profiles of a membrane system can be seen within the fibres. The relationships of the membrane systems observed to the fibrillar components have not been satisfactorily determined, and it is not known if there is a system equivalent to the T-system of vertebrates. The fibres are indistinctly striated exhibiting a wavy Z-line and each fibre is closely associated with the adjacent fibres, separated only by a folded sarcolemma. Each protrusion of the sarcolemma corresponds to an indentation in the sarcolemma of the neighbouring fibre. This may serve to hold the muscle fibres together.

E. Neuromuscular Junctions

Neuromuscular junctions in the crinoid have not been observed by light microscopy. Under the electron microscope, nerves are frequently seen in close proximity to the muscle fibres. These nerves pass between the muscle fibres at right angles to the longitudinal plane of the muscle (Fig. 40), but in the absence of evidence of junctional specialization these cannot be regarded as transmitting points. It has been difficult to determine precisely the exact point of innervation but several specialized features have been observed in certain areas of association between the nerves and the muscle fibres. These features are here, tentatively regarded as indicative of

neuromuscular junctions. The features in question are the presence of numerous clear vesicles in the nerve and concentrations of mitochondria in the muscle adjacent to the contact of the nerve fibre to the sarcolemma (Fig. 41). Mitochondria do occur elsewhere in the muscle but are more scattered than those observed near the neuromuscular junctions. Mitochondrial length is highly variable but generally averages approximately 1.5μ near the junctions. The membranes near the point of contact are relatively smooth, lacking the characteristic folding or thickening described in many neuromuscular junctions. The large clusters of clear vesicles at the junctions average 1500 Å in diameter and are similar to those seen throughout the whole aboral nervous system. So far vesicles with dense contents have not been seen in the nerves innervating the flexor muscle nor at the neuromuscular junction. The only descriptions of the fine structure of the neuromuscular apparatus in echinoderms are of the lantern retractor muscle of Echinus esculentus (Cobb and Laverack, 1966b) noted previously. The neuromuscular junctions observed in this echinoid resemble those found in Florometra except that the wing-like process passing from the muscle cell around the nerve ending has not been observed in Florometra.

The clear synaptic vesicles found at the neuromuscular junction possibly contain acetylcholine or an acetylcholine-like

substance. Although there is no direct evidence of acetylcholine being the transmitter substance in the crinoid, indirect evidence strongly indicates that this may be the case. A single test for cholinesterase on the flexor muscle using Gomori's acetylthiocholine method (Pearse, 1961) indicated that cholinesterase was present in the muscle. The reaction was not localized to any large extent but can be regarded as positive. A more selective reaction using such methods as the azo coupling method (Pearse, 1961) might be advantageous both from the standpoint of determining the exact nature of the transmitter substance and of determining the locality and number of motor endplates in the muscle. This was not attempted in the present study. An acetylcholine-like substance has been described in the holothurians (Bacq, 1935) and in the asteroids (Unger, 1962). High levels of acetylcholine, up to 100µg/gm. have been reported in the radial nerves of asteroids (Welsh, 1966). Such drugs as nicotine and atropine have also been shown to affect the muscular action in echinoderms (Welsh, 1966 and Moore, 1920) in the ways expected if the transmitter substance was acetylcholine. Thus there seems to be good evidence for cholinergic transmission in many echinoderms, including the crinoid.

F. Neurosecretion

Unger (1960, 1962) was the first person to describe neurosecretory cells in echinoderms, specifically in asteroids. He

was able to demonstrate a selective staining of numerous bipolar and multipolar neurons in the nerve ring and radial nerves in Asterias glaciales by the common neurosecretory stains. Fontaine (1962) further extended this to include the ophiuroids by describing three cell types in the motor ganglia of Ophiopholus aculeata, all containing a granular substance characteristic of neurosecretion. He also indicated that the substance could be traced for considerable distances out along the cell processes, suggesting axon transport as the principal means of transport. He was, however, unable to find evidence of terminal reservoirs or neurohemal organs. During preliminary observations on the aboral nerve in Florometra using paraldehyde fuchsin staining methods (Ewen, 1962) neurons containing deep purple Gomori positive inclusions were also shown to occur in the crinoid (Fontaine and Goyette, unpublished work cited by Welsh, 1966). These neurons are large bipolar and multipolar ganglion cells corresponding to those previously described throughout the aboral nervous system (Figs. 42 and 43). The Gomori-positive inclusions can be seen concentrated near the regions of the cell processes and can be traced for some distance (up to 60μ) out along these processes (e.g. Figs. 42 and 43). The diameter of these inclusions varies considerably but in the figures shown above, they average approximately .5 to .8μ. In addition to the Gomori inclusions the entire cytoplasm of the cell body also shows an intense background

staining to the neurosecretory stain. In slides prepared by routine procedures (e.g., Masson's trichrome method) these inclusions impart a granular appearance to the cytoplasm. In descriptions of these large ganglion cells in other crinoids frequent reference has been made to their granular appearance (Clark, 1921) and it is probable that the granules concerned represent the same material as stains in Florometra with the Gomori method.

In all areas where these ganglion cells have been found, this affinity for paraldehyde fuchsin can be demonstrated. In some individuals the amount of fuchsinophilic material is relatively high while in others it is so sparse that it is difficult to demonstrate.

The presence of cells exhibiting an intense affinity for neurosecretory stains has for many years been the sole criterion for neurosecretory activity. In view of this, the large ganglion cells occurring in the aboral nervous system in Florometra could be considered to be neurosecretory cells. However, paraldehyde fuchsin and other neurosecretory stains although fairly selective for neurosecretory material also stain lipofuscin pigment, glycogen accumulations, lysosomes, and mucopolysaccharides (Simpson et al., 1966). In recent years certain reservations have thus been expressed concerning the feasibility of recognizing neurosecretory products on the basis of staining alone. The presence of fuchsinophilic nerve cells in

Floremetra therefore only suggests the possibility of neurosecretion. To establish its existence definitely it would be necessary to show evidence of cyclical activity in the cells and to demonstrate a physiological effect of the secretions (Simpson et al., 1966). In this present study attempts have been made to provide such evidence.

Electron micrographs indicate that lipofuscin pigment is the only substance present that might be mistaken for neurosecretory material. Glycogen and mucopolysaccharides (recognizable by the characteristic ultrastructure) have not been found within the nerve and the total number of lysosomes occurring within the cell body is very small. Lipofuscin is certainly present throughout the nervous system, particularly in the older animals. As noted previously many of the nerve cells contain lipofuscin granules approximately 1.0μ in diameter which although somewhat larger than the majority of the presumed neurosecretory products could be mistaken for this neurosecretory material. If KMn04 oxidation is omitted in the Gormori method neither the background material nor the large granules stained, but the latter can still be distinguished by their natural yellow colour, whereas the former is not visible at all. The amount and distribution of the lipofuscin can also be determined by staining with Sudan black B. By this it has been established that,

while contributing to some extent to the fuchsinophilic staining, the lipofuscin granules are responsible for only a small part of the total staining. It is apparent that the intense background staining and a considerable proportion of the larger inclusions present within the nerve cell bodies represent a substance other than lipofuscin, thus presumed to be a neurosecretory product. Also as mentioned previously (page 27) electron microscopy shows lipofuscin granules to be present only in the cell body, whereas granules interpreted as neurosecretory occur along the nerve fibres as well as in the cell body (Figs. 42 and 43).

Although they are much smaller than the granules seen by light microscopy the only structures that can be reasonably equated with the neurosecretory material demonstrated by paraldehyde fuchsin staining are the vesicles with dense contents seen in Figure 17. Since there is a certain amount of alteration of the tissue during paraffin treatment the appearance of these granules might become altered and aggregate to form some of the larger granules seen with the light microscope. These dense core vesicles can be found both within the nerve cell body (Fig. 44) and out along the axon (Fig. 17) and it is probable that axon transport is involved in the distribution of the neurosecretory material. They have been found in all areas of the aboral nervous

sytem studied so far, namely the nerve ring, brachial nerves and the nerves in the gonadal pinnules. The concentration of these vesicles varies considerably in sections taken from different animals. Figure 45 shows the extent of this concentration. The amounts present bear no relation to the age of the individual or to the fixative used and it therefore appears likely that there is some cyclic activity or physiological variation leading to differences in concentrations of these vesicles in different animals. Animals were collected during the months of July, 1965 and May, 1966 and the differences were apparent regardless of the time of collection. So far the reason for the apparent differences in concentration has not been definitely established. It has, however, been observed that while most of the animals did not carry ripe pinnules at the time of collection some had mature gametes, apparently ready for shedding. Sections of the latter did show extremely large concentrations of the dense core vesicles in many parts of the nervous system indicating a possible role in the reproductive physiology. Similar vesicles could also be found along the fine nerve branches extending out among the collagen fibres of the gonadal pinnules (Figs. 46 and 47). Many nerve trunks in the locality of the eggs contained in the gonadal pinnules contain numerous dense core vesicles.

The exact nature of the substance within the dense core vesicles (which will now be referred to as neurosecretory vesicles) has not been definitely established. Their appearance of a dark centre surrounded by an electron lucid periphery is reminiscent of vesicles known to contain biogenic monoamines (DeRobertis, 1964; Clementi et al., 1966; Wood and Barrnett, 1963). However, tests for catecholamines and 5-HT using fluorescence analysis (Falck and Owman, 1965) failed to demonstrate the presence of these amines in the nerve ring in Florometra. A strong green fluorescence was observed in the tissue with or without paraformaldehyde treatment. Specificity tests using sodium borohydride failed to eliminate this fluorescence and indicated the presence of a primary fluorescence inherent in the tissue. Successful controls were run simultaneously with the aboral nerve, using rat adrenal medulla in these tests. Recalling the large variation in the concentration of the neurosecretory vesicles there is still a remote possibility that biogenic amines are present in Florometra but that the six nerve rings chosen for testing happened to be low in vesicle concentration. Conclusive evidence of the absence of biogenic amines would require that the tests be run concurrently with electron microscope observations of the same tissue.

IV. DISCUSSION

It is generally accepted that the echinoderms came from a bilateral ancestor and this is suggested particularly by the bilaterality of the larval stages. With their adoption of radial symmetry they have lost all trace of an anterior end. The nervous system, being based on this radial plan, appears to be in a rather low state of organization. Yet with the development of this radial plan a wide variety and a great number of moving parts for locomotion and other organismal activities have arisen, necessitating the evolution of a relatively complex co-ordinating system of nervous control and co-ordination of movement.

Ewer (1965) suggests that many of the co-ordinated activities are organized largely by ganglionic complexes which are immediately adjacent to the various effectors in question, which from an engineering point of view appears to be a rational type of organization. The many effectors respond independently, both to local stimuli and to more general stimulation as well. In order to bring these various action systems together in some co-ordinated pattern, specialized and integrating pathways have evolved within the nervous system. In the asteroids these pathways are contained in the radial nerves and circumoral nerve ring. A similar plan is found in the crinoid, the aboral nervous system serving to co-ordinate and transmit activities throughout the animal. The present study has dealt exclusively with this portion of the nervous system.

The aboral nervous system in the crinoids, is apparently unique in having a mixed composition of both afferent and efferent fibres (Smith, 1966), with the ability to order and regulate the major activities of the crinoid. In function it is comparable to ectoneural-hyponeural complex of the asteroids. The respective sensory and motor roles of the latter complex are, therefore, thought to be combined in the aboral nervous system of the crinoids and this system constitutes the main conducting component. Little, however, is known about the nervous system of the crinoid beyond its gross morphology and for that matter knowledge of the nervous function generally in the phylum is confined to a few preliminary and exploratory investigations.

Since the special staining methods for showing nerve connections have proven unsatisfactory in the echinoderms, the neuron-to-neuron connections have not been traced in the crinoids and have only been inferred in the asteroids by Smith (1965). From physiological findings by Marshall (1884) and Moore (1924), confirmed in the present study, it appears that the pinnules, podia, portions of whole arms and whole arms, possess the ability to respond independently of the neighbouring parts. They are capable of their own independent movements. The intact nerve ring is necessary for the co-ordination of the movement of these numerous independent parts with that of adjacent effectors and with those in other areas of the body. A mild stimulus to the arms, whether intact or amputated, produces a localized response adjacent to the stimulated area. This response is usually a localized

flexion of the arm or covering of the stimulated area by the pinnules. Increasing the stimulus strength leads to spreading of the response in all directions from the point of the stimulus. Further intensification of the stimulus causes a spreading of the response successively to the arm which arises from the same base as the arm stimulated, to the adjacent arms and finally, with a stimulus of sufficient strength, to the whole organism, which then escapes by swimming. From the experiments conducted by Marshall and Moore, the aboral nerve ring (or more probably the pentagonal commissure), as well a functioning as a link between the arms also serves as a co-ordinating centre for locomotion and maintenance of muscle tone. On removal of the nerve ring the arms remain rigid presumably because there is no muscle tone being exerted against the ligaments. If the nerve ring is severed the arms are still capable of a swimming movement but lack co-ordination.

The various levels of integration inferred from these experiments are reflected in the morphological appearance of the aboral nervous system as one passes down the nerve cord to the aboral nerve ring. The presence of numerous nerve cells scattered along the length of the nerve cords suggest the possibility of local integrative or relay circuits along the length of the nerve cord. This mechanism would provide for the local autonomy that is present in the various moving parts through short fibre pathways within the arms. It is known that a decremental spread of excitation occurs in the echinoderm nervous system and the total number of action systems affected

would be dependent on the strength of the stimulus. If the stimulus were of sufficient strength the impulse could reach the other member of the arm pair through the transverse commissure or pass to the adjacent arms through the nerve ring. The significance of the chiasma in the integrative events of the nervous system has not been determined.

As mentioned previously, if the stimulus is strong enough the crinoid will respond by swimming. This complex activity in which alternating flexions occur between the arms (the alternate arms move in the same phase but opposite to their neighbours) would require a precise mechanism of co-ordination. The experiments on the nerve ring by Marshall and Moore showed too that the nerve ring is important in co-ordination and maintenance of muscle tone. Aggregation of nerve cell bodies are found at the bases of the nerve trunks leading to the brachial nerve cords, which suggests that these may be the pacemaker centres involved in co-ordination of swimming, etc. and in the maintenance of muscle tone. Similar concentrations of cell bodies have been shown in the asteroids to occur at the junctions of the radial nerves to the circumoral nerve ring. Smith (1965) suggests the direction of movement is dependent on the dominance of one of these centres over the others. The direction of movement of the animal is presumed to be determined wholly by the activity of nerve centres at the base of the leading arm. The muscle tone could also be maintained through a sustained excitation originating from the same centres.

Many lines of evidence including those mentioned above indicate that the aboral nervous system has a high degree of complexity and is only partially understood. According to Bullock and Horridge (1965) in all animals with a central nervous system there is a division of its tissue into two zones, fibrous and nucleated. In invertebrates the two zones are conveniently called the rind and the core; the nerve cell bodies are typically gathered in a layer on the outside of the ganglion or cord leaving a central mass of nerve fibres free of cell bodies. The cell rind is free of synaptic fields; the synaptic fields as well as long pathways being restricted to the fibrous core. This is in contrast to the vertebrates where the inside gray matter contains nerve cell bodies and nerve endings, both axonal and dendritic and thus synaptic fields. The white matter consists simply of axons en route to distant points. The fibre core is differentiated except in the lowest invertebrates into tracts and neuropile; tracts are fibres en route without endings or synapses; the neuropile is a plexus of axonal terminations, dendrites and synapses. The degree of differentiation of the neuropile is a sensitive indicator of the level of central nervous specialization, the higher forms having a more complete separation of the tracts from the neuropile. In the annelids and lower invertebrates the separation is incomplete.

The aboral nerve ring in Florometra shows little of this differentiation. Nerve cell bodies are scattered throughout the ring, although there is a tendency for cell bodies to concentrate around the

the cortex, and at the bases of the nerve trunks. The nerve cords of the arms, however, show distinct differentiation into cell rind and fibrous core. The nerve cords possess a layer of cell bodies, some projecting out into the calcified connective tissue. Immediately beneath the rind lies the neuropile, a tangled network of fibres devoid of cell bodies corresponding to the 'diffuse neuropile' in the sense used by Maynard (1962). In this category he has included the posterior ganglia of most higher invertebrates and many peripheral invertebrate ganglia. This is probably the area containing the cell arborizations and synaptic fields. So far synapses have not been observed directly on the cell soma in the crinoid suggesting that the synapses are outside the cell body regions (i.e. in the neuropile). This region of the nerve cords is probably the region of the integrative events for that given region of the arm. Interior to the neuropile is an additional cell body concentration lying around the central tract of larger parallel nerve fibres. These cell bodies are arranged in chains along the length of the nerve cords. The central fibre tract consists of nerve fibres en route from the nerve ring and possibly provides for a more rapid conduction to the other areas of the nervous system. Smith (1966) upon consideration of the rapidity of arm movements advocated the presence of large nerve fibres and of nerve cells possibly arranged serially. In Florometra the morphological appearance of the nerve cords supports such an hypothesis. The transmission of the impulse within the nerve cord would be provided for by fibre pathways of varying fibre diameter.

The ultrastructural appearance of the aboral nervous system is similar in many respects to that of other invertebrate nervous systems. The nerves are unmyelinated which is generally true of most invertebrate phyla and contain numerous vesicles, both clear and electron-dense, neurotubules, and neurofilaments. Many of these features are also common to the vertebrates. In addition to these common feature certain nerve elements have been modified for a more special role in nervous function. This will be discussed in the following section on neurosecretion.

Neurosecretion

Without further histochemical and biochemical analysis it cannot be proved definitely that neurosecretory activity is present in the aboral nervous system in Florometra. Nevertheless, it seems probable that (from a morphological and histochemical standpoint) certain nerve cells within the aboral nervous system possess a degree of neurosecretory activity, producing a substance of protein or polypeptide nature. It is on this assumption that the present discussion is based.

It has previously been established (page40) that the aboral nervous system contains numerous large bipolar and multipolar neurons that exhibit a high affinity for the neurosecretory stain, paraldehyde fuchsin. The substance responsible for this staining is present throughout the cell body as fine granules, which impart an intense background staining to the

cytoplasm of the perikaryon. Accumulations of larger, more intensely staining granules are also present within the cell body and can be traced for some distance along the nerve fibres. These fuchsinophilic cells could be found throughout the nervous system with heavier concentrations near the cortical areas of the nerve ring and brachial nerve cords and the bases of the primary and secondary nerve trunks.

Until recently many descriptions of neurosecretion have been based solely on the demonstration of nerve cells showing an affinity to such neurosecretory stains as paraldehyde fuchsin. The capabilities of these stains reacting with other non-neurosecretory materials in many instances have been overlooked. The presence of these nerve cells can only tentatively indicate the possibility of neurosecretion and should be accompanied by further tests and observations before any definite conclusions are reached.

The rationale behind neurosecretory staining is not clearly understood but evidence suggests that in some cases the hormonal material or possibly the carrier substance is a protein with both weakly acidic and weakly basic tendencies and in many cases rich in cystine (Sumner, 1965). The staining method involves the alteration of this protein material to strong acids by oxidation and the subsequent linkage between the paraldehyde fuchsin and the acidic groups. Other reactive components such as the aldehydes in acid mucopolysaccharides also stain with paraldehyde fuchsin but do not require prior oxidation. In the case of Florometra where sections were

not subjected to oxidation, in this case by acidified potassium permanganate, the cell bodies failed to show the intense staining reaction towards the paraldehyde fuchsin shown by the same tissue that had had prior oxidation. This clearly indicates that the neurons contain a substance requiring mild oxidation in order to react with the paraldehyde fuchsin. This suggests a protein or polypeptide substance. As was noted previously (page 41) lipofuscin, which can occur throughout the nerves stains in the same way as the neurosecretory product, but can only be responsible for a minor part of the reaction seen. The possibility ot non-polar bonding between the dye and the tissue aldehydes should be mentioned. If this were taking place staining would presumably occur even if oxidation was omitted, but this was not found to be the case. Finally, oxidation might liberate other potential reactive groups within the cytoplasm but there appears to be no reports of confusion arising from this source in the literature. In spite of strong evidence of the presence of neurosecretion in Florometra caution must still be exercised in the interpretation of the distribution of neurosecretory material to avoid confusion with the large amounts of lipofuscin pigment present particularly when it occurs as isolated granules within the cytoplasm of the cells. The presumed neurosecretory material for the most part borders on the limits of resolution of the light microscope and observations should be coupled with electron microscope studies.

Electron microscope observations have shown that the most probable structures involved in neurosecretion are the dense-core or neurosecretory vesicles described on page 43. These neurosecretory vesicles can be found in the cell bodies and out along the nerve fibres, a distribution similar to that of the fuchsinophilic granules described in the light microscope. The production of these vesicles appears to take place within the nerve cell body and the most probable means of transport is out along the nerve fibres. The nerve cells containing these granules generally show signs of protein synthesis such as a prominent nucleolus and rough endoplasmic reticulum that varies with the amount of dense core vesicles that are present. An increase in the concentration of these vesicles is usually associated with an abundance of endoplasmic reticulum. The core size also varies with the apparent secretory activity, the core almost filling the entire vesicle whenever large amounts of vesicles are present. The vesicular diameter cannot be used as a criterion for recognizing neurosecretory vesicles (a criterion applied with the vertebrates) since all types of vesicles in the crinoid seem to be considerably larger than those found in vertebrates.

The appearance of these neurosecretory vesicles gave rise to questions concerning the possibility that these vesicles contain a catecholamine or 5-HT. It will be recalled that tests failed to demonstrate any major monoamine in the aboral nerve but the remote possibility still existed that low concentrations happened to have been present in the specimens used for analysis. Ostlund (1954) using pharmacalogical methods also failed to

show any evidence of adrenaline-like substance in whole body extracts of sea cucumber, sea urchin, or brittle stars. However, low quantities of monoamines could go unnoticed with this type of determination. Welsh and Moorhead (1960) found low quantities of serotonin (5-HT) in whole or nearly whole extracts of certain asteroids, holothurians, and echinoids. Serotonin was also found in the circumoral region (containing the nerve ring) of Thyone briareus but was not definitely localized in the nerve ring. Radial nerves of Asterias forbesi have been assayed, yielding relatively small amounts of serotonin (a few tenths of a microgram per gram of wet nerve) (Welsh and Cottrell, unpublished, cited by Welsh, 1966). Amine oxidase, important in the catabolism of catecholamines has been reported in the digestive glands of four species of asteroids and in the intestine of two species of echinoids (Blaschko and Hope, 1957) but not detected in three species of holothurians. Unger (1962) in his chromatographic analysis of radial nerve extracts was unable to demonstrate a substance in the elluate corresponding to those of the known Rf values of adrenalin, nor-adrenalin, and serotonin. From the preceeding account it is apparent that the presence of monoamines in the echinoderms cannot be ruled out but there is little evidence of their participation as a neurotransmitter substance in the nerves of any echinoderms and in the case of Florometra it is probable that they are completely absent from the nervous system. Thus it can be stated with reasonable certaintly that the substance within these vesicles can be regarded as neurosecretory material, functioning as a neurohumor.

The existence of an endocrine organ in the echinoderms has not yet been observed and the most probable means of endocrine activity is vested in the nervous system. The part that the presumed neurosecretory material plays in the biology of the crinoid is still undetermined. It has, however, been noted that the total concentration of dense core vesicles varies between individuals. Collections were made during the breeding season, assuming that this extends from late May to early August as reported by Mortensen (cited by Boolootian, 1966) for Florometra. This individual variation in vesicle concentration may be related to their phase of reproductive activity within the breeding period, some having shed their gametes, others still bearing them. Where gametes were still present, neurosecretory vesicles were invariably present in high concentrations. It was first shown by Chaet and McConnaughy (1959), Chaet and Rose (1961), and Chaet (1964) that the radial nerves of starfish contain a substance inducing the shedding of gametes. Unger (1962) also mentioned the release of sperm upon injection of radial nerve extracts into the coelomic cavities of starfish. Since then Imlay and Chaet (1965) and in more detail Kanatani and Noumura (1962) have shown that the presence of neurosecretory material in the radial nerves is correlated with the presence of gamete shedding substance. This material is thought to be identifiable with granules of 1μ diameter seen by light microscopy. Histochemical and biochemical tests indicate that the active material may be a protein or polypeptide. Chaet (1966) has suggested that a "contraction factor" is present in the shedding substance

causing the ovary to contract several minutes before eggs are released. Release occurs after a latent period of 30 to 35 minutes and is dependent upon calcium in the surrounding medium. Chaet and co-workers have also postulated that an inhibitor substance is present in radial nerve extracts. This factor ("shedhibin") is found only in ripe animals and overrides the effect of the shedding substance when the shedding substance is injected experimentally in above threshhold amounts.

Without knowing the precise distribution of these electron dense vesicles and so being able to establish a definite target site, we can only speculate about their influence on reproduction but it seems probable that these vesicles contain a substance active in inducing gamete shedding. The vesicles have been traced to the vicinity of the eggs in ripe pinnules, occurring both in the pinnule nerves and their branches. Release of the hormonal substance could occur within the vicinity of the ovary, acting directly on the ovary itself. Chaet's observations on natural spawning have indicated a release from the oral surface of the radial nerve directly into the sea water as well as transport to the gonads through the coelomic cavities. It is known that simultaneous spawning occurs in whole populations of crinoids to the extent that it can be timed precisely each year. It occurs synchronously in both whole animals and amputated arms, even when kept under constant conditions in the laboratory (Boolootian, 1966). This would require a precise mechanism of control of the gonads and possibly the

interaction between neighbouring animals by means of diffusable substances released into the water. This neurosecretory activity may represent a biological clock, timing the release of the gametes.

The present study has succeeded only in touching the surface of the vast amount yet to be known about the biology of the crinoids and, indeed the echinoderms as a whole. It has shed some light on the complex structure and possible functions of the nervous system, while at the same time opening up new areas begging inquiry. The nature and role of neurosecretion in the crinoids is in need of further study. A definite determination of its chemical composition, of its total distribution, and of the exact location of target sites together with precise information on seasonal variation are some of the factors most in need of further study.

V. LITERATURE CITED

Bacq, Z.M. 1935. Occurrence of unstable cholinesters in invertebrates. Nature, Lond. 136:30-31.

Bennett, H.S., and J.H. Luft. 1959. S-collidine as a basis for buffering fixatives. J. biophys. biochem. Cytol. 6:113-114.

Blaschko, H., and D.B. Hope. 1957. Observations on the distribution of amine oxidase in invertebrates. Archs Biochem. Biophys. 69:10-15.

Boltt, R.E., and D.W. Ewer. 1963. Studies on the myoneural physiology of Echinodermata. V. The lantern retractor muscle of Parechinus: responses to drugs. J. exp. Biol. 40:727-733.

Boolootian, R.A. 1966. Reproductive physiology. p. 561-613. In R.A. Boolootian (Ed.). Physiology of Echinodermata. John Wiley and Sons, New York.

Bullock, T.H., and G.A. Horridge. 1965. Structure and Function in the nervous systems of invertebrates. 1. p. 49-58. W.H. Freeman and Co., San Francisco.

Carleton, H.M., and R.A.B. Drury. 1957. Histological technique. Oxford Univ. Press. London 3rd Ed.

Chaet, A.B. 1964. Shedding substance and "shedhibin" from the nerves of the starfish, Patiria miniata. Am. Zool. 4:407.

Chaet, A.B. 1966. The gamete shedding substances of starfishes: A physiological-biochemical study. Am. Zool. 6:263-271.

Chaet, A.B., and R.W. McConnaughy. 1959. Physiologic activity of nerve extracts. Biol. Bull. 117:407.

Chaet, A.B., and R.A. Rose. 1961. Further studies on the gamete 'shedding substance' from radial nerves. Biol. Bull. 121:385-386.

Clark, Austin Hobart. 1921. A monograph of the existing crinoids. Bull. U.S. Nat. Mus. 1(2):320-337.

Clementi, F., P. Mantegazza and M. Botturi. 1966. A pharmacologic and morphologic study on the nature of the dense core granules present in the presynaptic endings of sympathetic ganglia. Int. J. Neuropharmacol. 5:281-285.

Cobb, J.L.S., and M.S. Laverack. 1966a. The lantern of Echinus esculentus (L.) II. Fine structure of hyponeural tissue and its connections. Proc. Roy. Soc. Lond. Ser. B. 164:641-650.

Cobb, J.L.S., and M.S. Laverack. 1966b. The lantern of Echinus esculentus (L.) III. Fine structure of the lantern retractor muscle and its innervation. Proc. Roy. Soc. Lond. Ser. B. 164:651-658.

Corrodi, H., N.A. Hillarp and G. Jonsson. 1964. Fluorescence methods for the histochemical demonstration of monoamines. 3. Sodium borohydride reduction of the fluorescent compounds as a specificity test. J. Histochem. Cytochem. 12:582-586.

Davenport, H.A. 1960. Histological and histochemical technics. W.B. Saunders Co.,Philadelphia.

DeRobertis, E.D.P. 1964. Histophysiology of synapses and neurosecretion. Vol. 20. International series of monographs on pure and applied biology. P. Alexander and Z.M. Bacq (Eds.). The Macmillan Co., New York.

Dudley, P., and P. Dunlap. Unpublished procedure on Epon embedding techniques.

Ewen, A.B. 1962. An improved aldehyde staining technique for neurosecretory products in insects. Trans. Am. Microsc. Soc. 81(1):94-96.

Ewer, D.W. 1965. Networks and spontaneous activity in echinoderms and platyhelminthes. Am. Zool. 5:563-572.

Falck, B., and C. Owman. 1965. A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. Acta. Univ. lund. Section II, No. 7:1-23.

Fontaine, A.R. 1962. Neurosecretion in ophiuroid, Ophiopholus aculeata. Science 138:908-909.

Hamann, O. 1889. Anatomie and Histologie der Crinoiden. Jena. Z. Naturw. 23:233-388.

Hodgman, C.D., R.C. Weast and S.M. Selby. 1960. Handbook of chemistry and physics. p. 2500. Chemical Rubber Co., Cleveland.

Hyman, L.H. 1955. The Invertebrates. IV. Echinodermata. McGraw-Hill, New York.

Imlay, M.J., and A.B. Chaet. 1965. Microscopic observations of gamete shedding substance in starfish radial nerves. Fed. Proc. 24:129 (abst.)

Kanatani, H., and T. Noumura. 1962. On the nature of active principles responsible for gamete-shedding in the radial nerves of starfishes. J. Fac. Sci. Tokyo Univ. 9:403-416.

Kawaguti, S. 1964a. Electron microscopy on the intestinal wall of the sea cucumber with special attention to its muscle and nerve plexus. Biol. J. Okayama Univ. 10:39-50.

Kawaguti, S. 1964b. Electron microscopic structures of the podial walls of an echinoid with special references to the nerve plexus and the muscle. Biol. J. Okayama Univ. 10:1-12.

Kawaguti, S., and Y. Kamishima. 1965. Electron microscopy on the spine muscle of the echinoid. Biol. J. Okayama Univ. 11:31-40.

Lane, N.J. 1966. The fine-structural localization of phosphatases in neurosecretory cells within the ganglia of certain gastropod snails. Am. Zool. 6:139-157.

Luft, J.H. 1961. Improvements in epoxy resin embedding methods. J. biophys. biochem. Cytol. 9:409-414.

Marshall, A.M. 1884. On the nervous system of Antedon rosaceus. Quart. J. Microsc. Sci. 24:507-548.

Maynard, D.M. 1962. Organization of neuropile. Am. Zool. 2:79-96.

McManus, J.F.A. 1946. The demonstration of certain fatty substances in paraffin sections. J. Path. Bact. 58:93-95.

Moore, A.R. 1920. The action of strychnine and nicotine on the neuromuscular mechanism of Asterias. J. gen. Physiol. 2:201-204; 319-324.

Moore, A.R. 1924. The nervous mechanism of co-ordination in the crinoid Antedon rasaceus. J. gen. Physiol. 6:281-288.

Ostlund, E. 1954. The distribution of catecholamines in lower animals and their effect on the heart. Acta. Physiol. scand. 31, Suppl. 112:1-67.

Palay, S.L., and G.E. Palade. 1955. The fine structure of neurons. J. biophys. biochem. Cytol. 1:69-88.

Palay, S .L. 1960. The fine structure of secretory neurons in the preoptic nucleus of the goldfish (Carassius auratus). Anat. Rec. 138:417-444.

Pantin, C.F.A. 1962. Notes on microscopical technique for zoologists. Cambridge Univ. Press.

Pearse, A.G. 1961. Histochemistry, theoretical and applied. J. and A. Churchill Ltd., London, 2nd Edition.

Peters, A. 1955a. Experiments on the mechanism of silver staining. I. Impregnation. Quart. J. Microsc. Sci. 96:84-102.

Peters, A. 1955b. A general purpose method of silver staining. Quart. J. Microsc. Sci. 96:323-328.

Pople, W., and D.E. Ewer. 1954. Studies on myoneural physiology of echinodermata. 1. Pharyngeal retractor of Cucumaria. J. exp. Biol. 31:114-126.

Richardson, K.C., L. Jarett and E.H. Finke. 1960. Embedding in Epoxy Resins for ultra thin sectioning in electron microscopy. Stain Technol. 35:313-323.

Sabatini, D.D., K. Bensch and R.J. Barrnett. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. 17:19.

Simpson, L., H.A. Bern and R.S. Nishioka. 1966. Survey of evidence for neurosecretion in gastropod molluscs. Am. Zool. 6:123-138.

Smith, J.E. 1937. On the nervous system of the starfish Marthasterias glacialis. Phil. Trans. Roy. Soc. Ser. B. 227:111-173.

Smith, J.E. 1950. The motor nervous system of the starfish, Astropecten irregularis (Pennant) with special reference to the innervation of the tube feet and ampullae. Phil. Trans. Roy. Soc. Series B, 234:521-558.

Smith, J.E. 1965. Echinodermata. p. 1519-1558. In T.H. Bullock and G.A. Horridge. 1965. Structure and function in the nervous systems of invertebrates. II. Freeman and Company, San Francisco.

Smith, J.E. 1966. The form and function of the nervous system. p. 503-511. In R.A. Boolootian (Ed.). Physiology of Echinodermata. John Wiley and Sons, New York.

Smith, R.E., and M.G. Farquar. 1966. Lysosome function in anterior pituitary gland. J. Cell Biol. p. 31:319-347.

Sumner, Barbara, E.H. 1965. A histochemical study of aldehyde fuchsin staining. J. Roy. Microsc. Soc. 84:(3)329-338.

Unger, H. 1960. Neurohormone bei Seesternen. (Marthasterias glaciatis). Symp. Biol. Hungarica. 1:203-207.

Unger, H. 1962. Experimentelle und histologische Untersuchungen über Wirkfaktoren aus dem Nervensystem von Asterias (Marthasterias) glacialis (Asteroidea; Echinodermata). Zool. Jb. Physiol. 69:481-536.

Venable, J.H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25:407-408.

Watson, M.L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. biophys. biochem. Cytol. 4:475-478.

Welsh, J.H., and M. Moorhead, 1960. The quantitative distribution of 5-hydroxytryptamine in the invertebrates, especially in their nervous systems. J. Neurochem. 6:146-169.

Welsh, H. 1966. Neurohumors and neurosecretion. p. 545-560. In R.A. Boolootian (Ed.). Physiology of Echinodermata. John Wiley and Sons, New York.

Wood, J.G., and R.J. Barrnett. 1964. Histochemical demonstration of norepinephrine at a fine structural level. J. Histochem. Cytochem. 12:19 7-209.

VI. EXPLANATION OF FIGURES

Figures 1, 35 and 36 are drawings within the text; Figures 2 - 10, 29 - 32, 37, 38, 42 and 43 are light micrographs; and the rest are all electron micrographs. In all cases originals were made, labelled and then reproduced photographically.

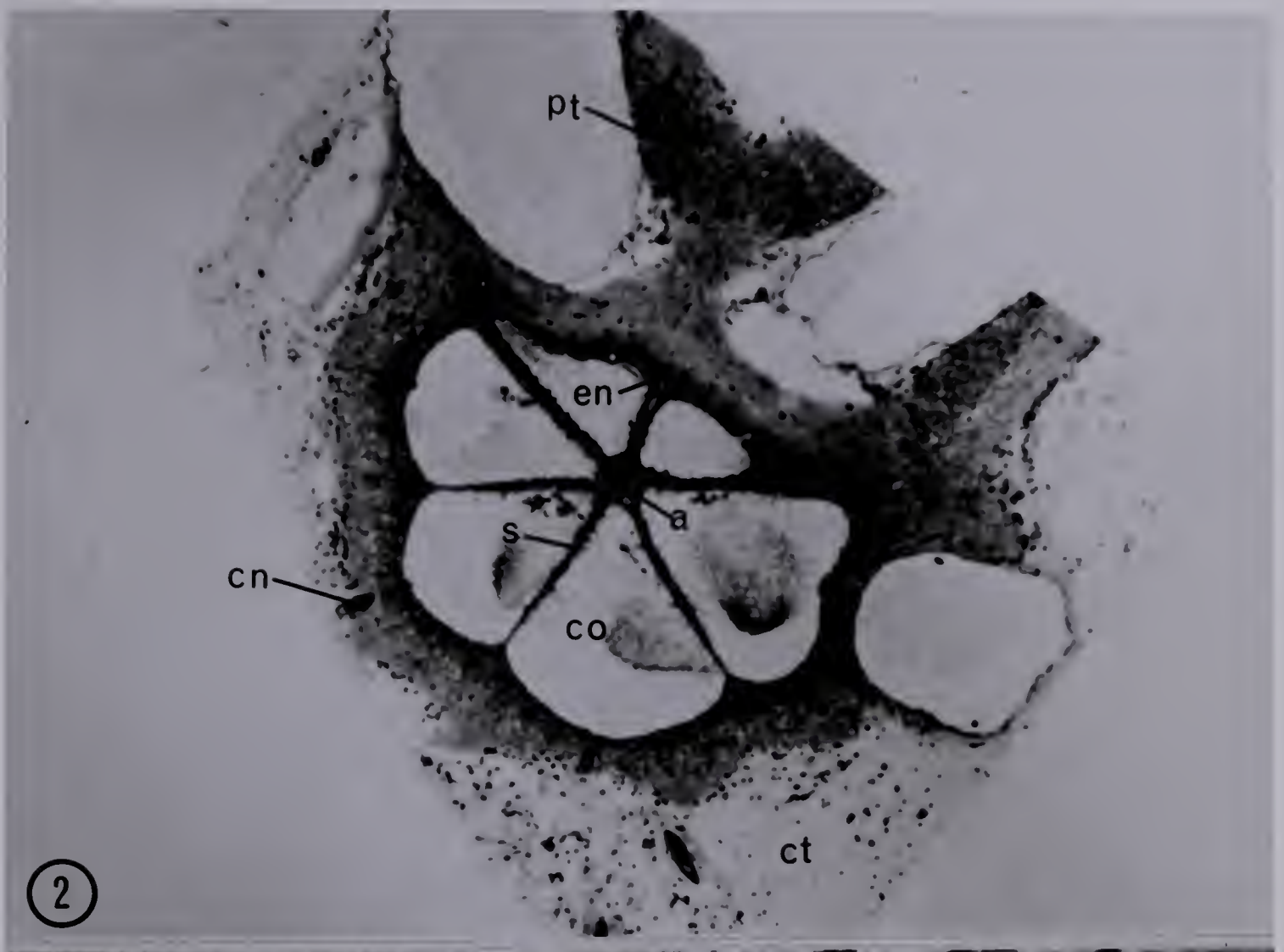
Fig. 2. An oblique section of the aboral nerve ring, showing its gross morphology and its relationship to the chambered organ (co). Section includes the region of the cirral nerves (cn) and the primary nerve trunk (pt). Compare with Fig. 1, text.

s, septa; a, axial gland; en, endothelial lining of the chambered organ; ct, calcified connective tissue of the calyx.

Glutaraldehyde/osmium fixation, Epon embedding and Richardson's staining. X 750.

Fig. 3. A light micrograph of the large bi-, and multipolar nerve cells located near the base of the primary nerve trunk. Note the paucity of chromatin and the prominent nucleolus within the nucleus. Also note the fine granular appearance of the cytoplasm with additional larger granules occurring within the cell body and out along the cell processes.

Susa fixation, paraffin embedding and paraldehyde fuchsin staining. X 1,500.


pt
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cn
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3

Fig. 4. Light micrograph of the aboral nerve ring showing the clusters of lipofuscin pigment (lp).

Osmium/S-collidine fixation, Epon embedding and Richardson's staining. X 1,500.

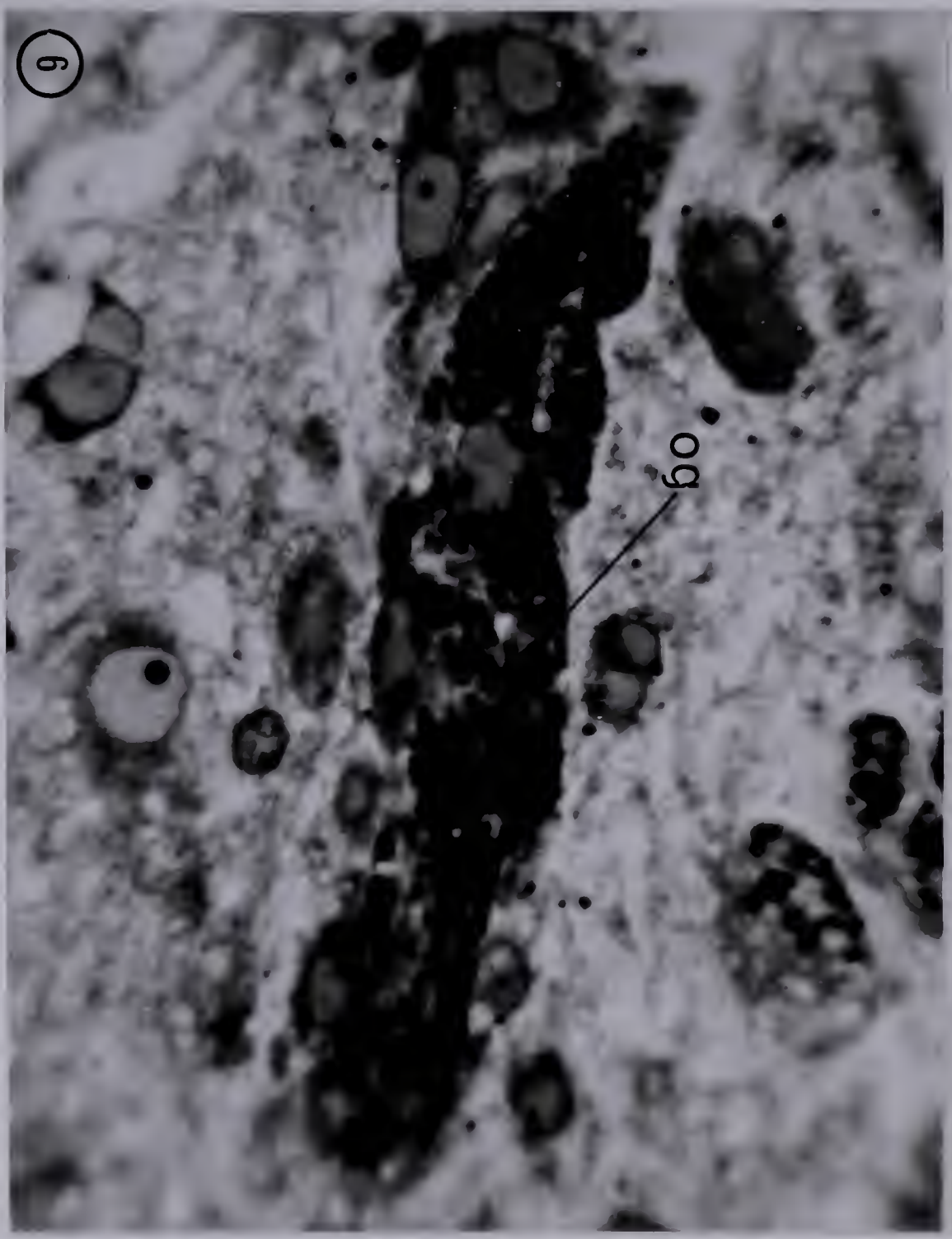
Fig. 5. A fresh preparation of the aboral nerve ring showing the distribution of lipofuscin pigment within the nerve ring. Heavier concentrations appear to correspond to the nerve cell body concentrations (arrows).

ab, aboral nerve ring; pt, primary nerve trunk leading to the arms; cx, calyx.

Aboral view. X 100.

Fig. 6. A light micrograph of non-nervous cells of unknown nature that contain large oval granules (og). These cells can be found scattered throughout the nervous system. X 4,500.

Osmium/S-collidine fixation, Epon embedding and Richardson's staining.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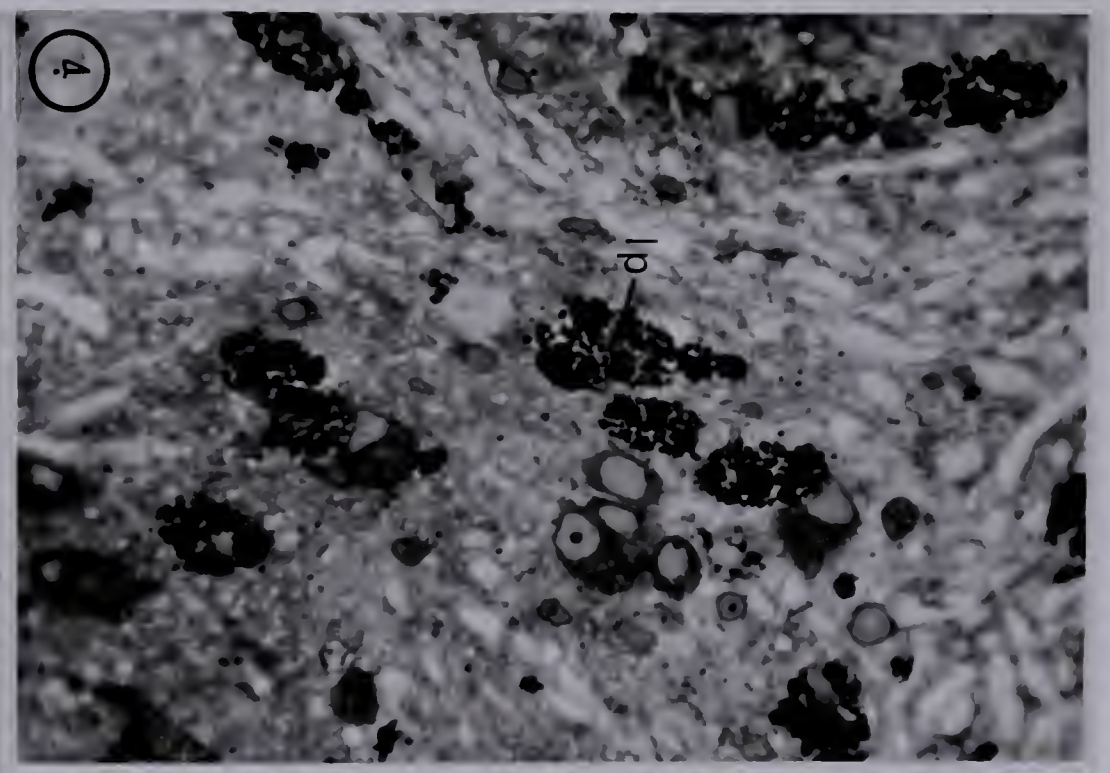
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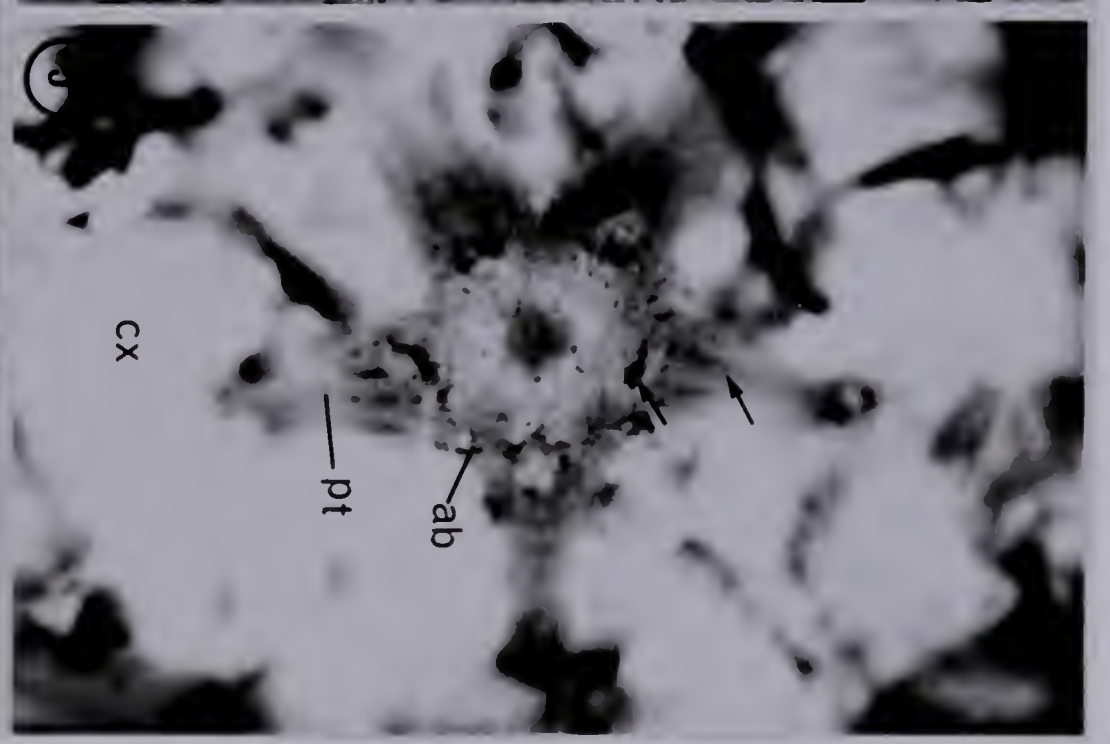
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Fig. 7. Endothelial lining of the chambered organ in the region of the cirral canals.

en, endothelium; co, chambered organ; cc, entrance of the cirral canals into the chambered organ. X 1,200.

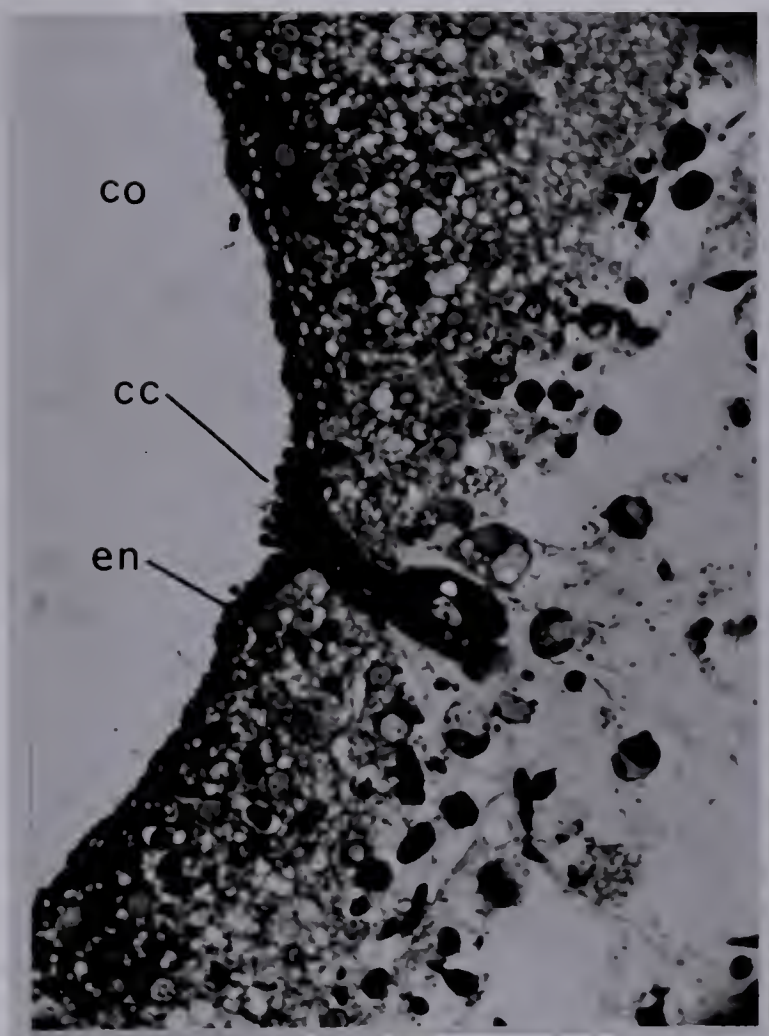
Fig. 8. Endothelial cells of the chambered organ in the region of the bases of the primary nerve trunks. Section shows the two cell types present (a, b). Vacuoles occur within the cytoplasm of the endothelial cells (v), with some containing a substance showing metachromasia to azure 2 (mv). X 1,2 00.

Fig. 9. A light micrograph of the chambered organ septa and portion of the axial gland. The septa are also lined with vacuolated cells secreting the metachromatic substance that is found within the cavities of the chambered organ, as numerous spheres or a fine network.

s, septa; co, chambered organ; a, portion of axial gland; sp, metachromatic spheres. X 1,200.

Fig. 10. A higher magnification of a septum shown in Fig. 9. X 3,000.

Glutaraldehyde/osmium fixation, Epon embedding and Richardson's staining.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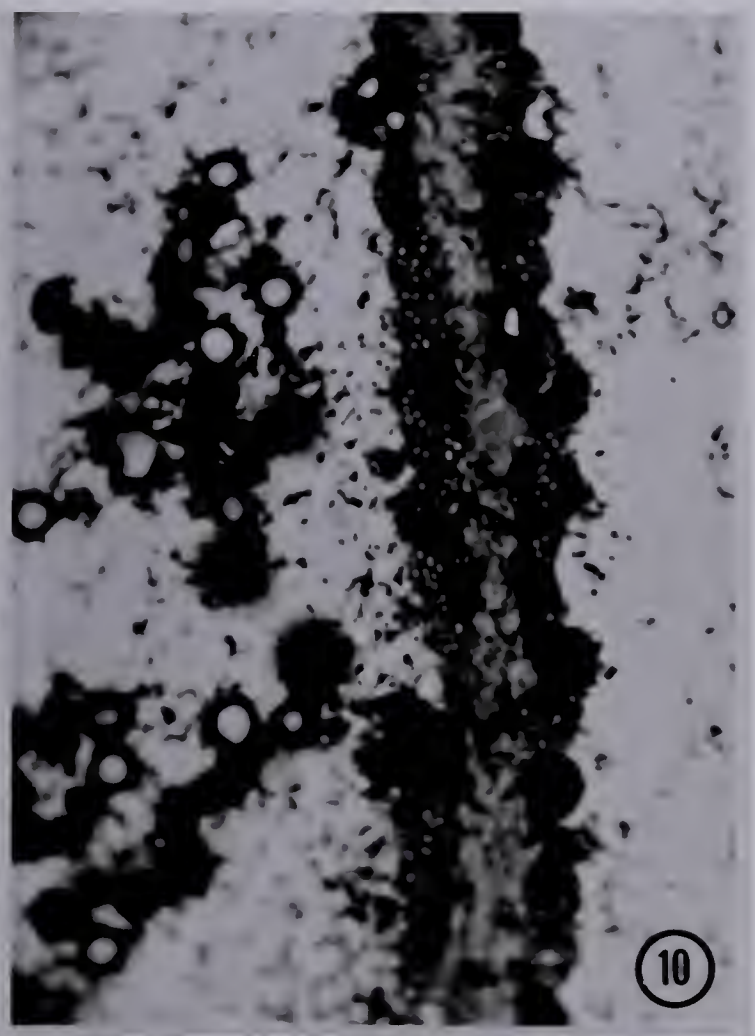
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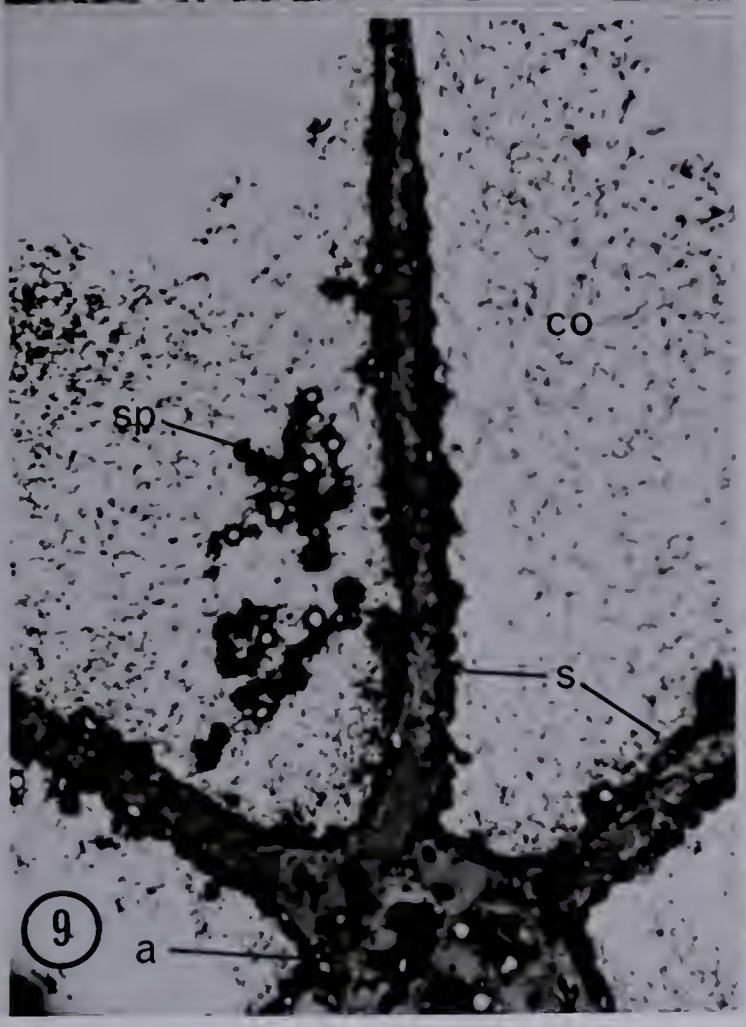
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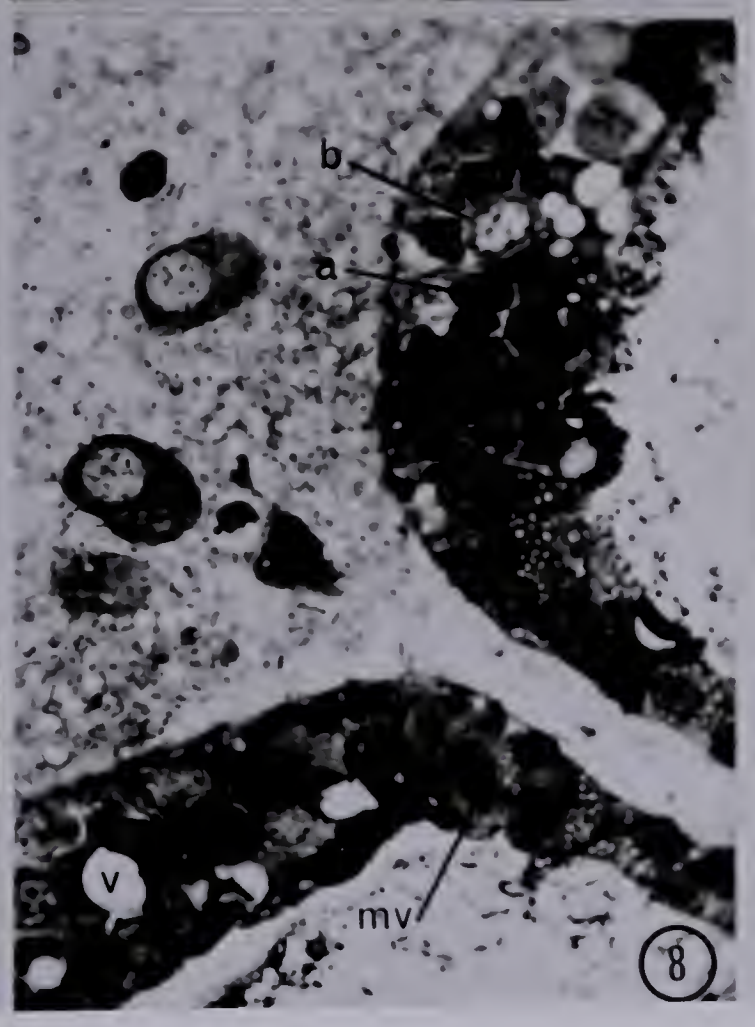
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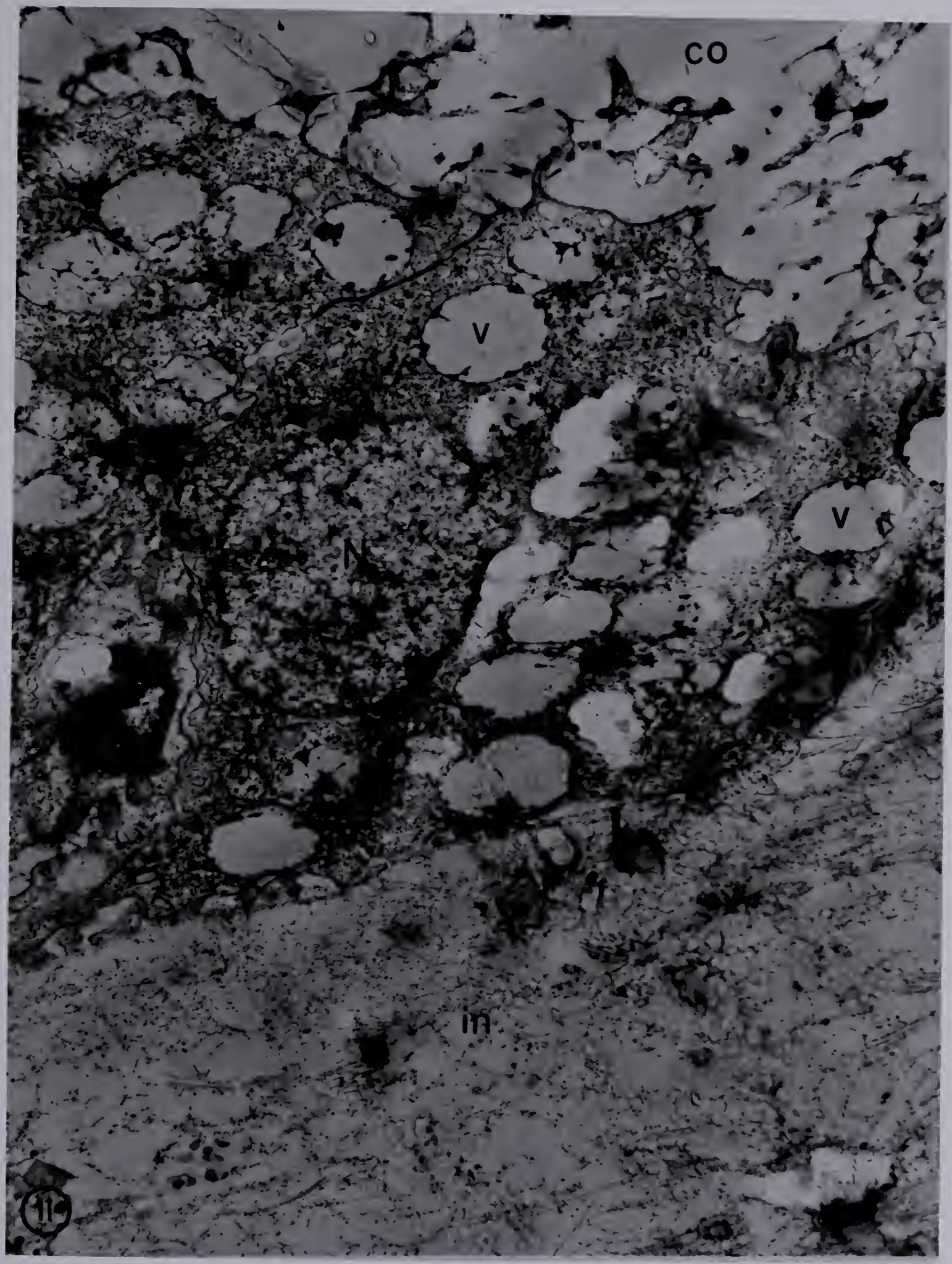
v
mv
8

Fig. 11. An electron micrograph of the vacuolated cells lining the septa.

N, nucleus; v, vacuoles; co, chambered organ; in, interior region of the septum.

Glutaraldehyde/osmium fixation, Epon embedding and lead citrate staining. X 15,000.


CO
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V
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Fig. 12. A cross section of the aboral nerve ring showing the typical appearance of the neuropile. The neuropile is composed of many interwoven unmyelinated nerve fibres with nerve cells interspersed among the nerve fibres. Note the collateral fibres running throughout the tissue.

N, nucleus of a nerve cell; nu, nucleolus; m, mitochondrion; l, lipid body; cb, cell body; nf, nerve fibre; cl, collateral fibre.

Osmium/S-collidine fixation, Epon embedding and uranyl acetate-lead citrate staining. X 7,500.

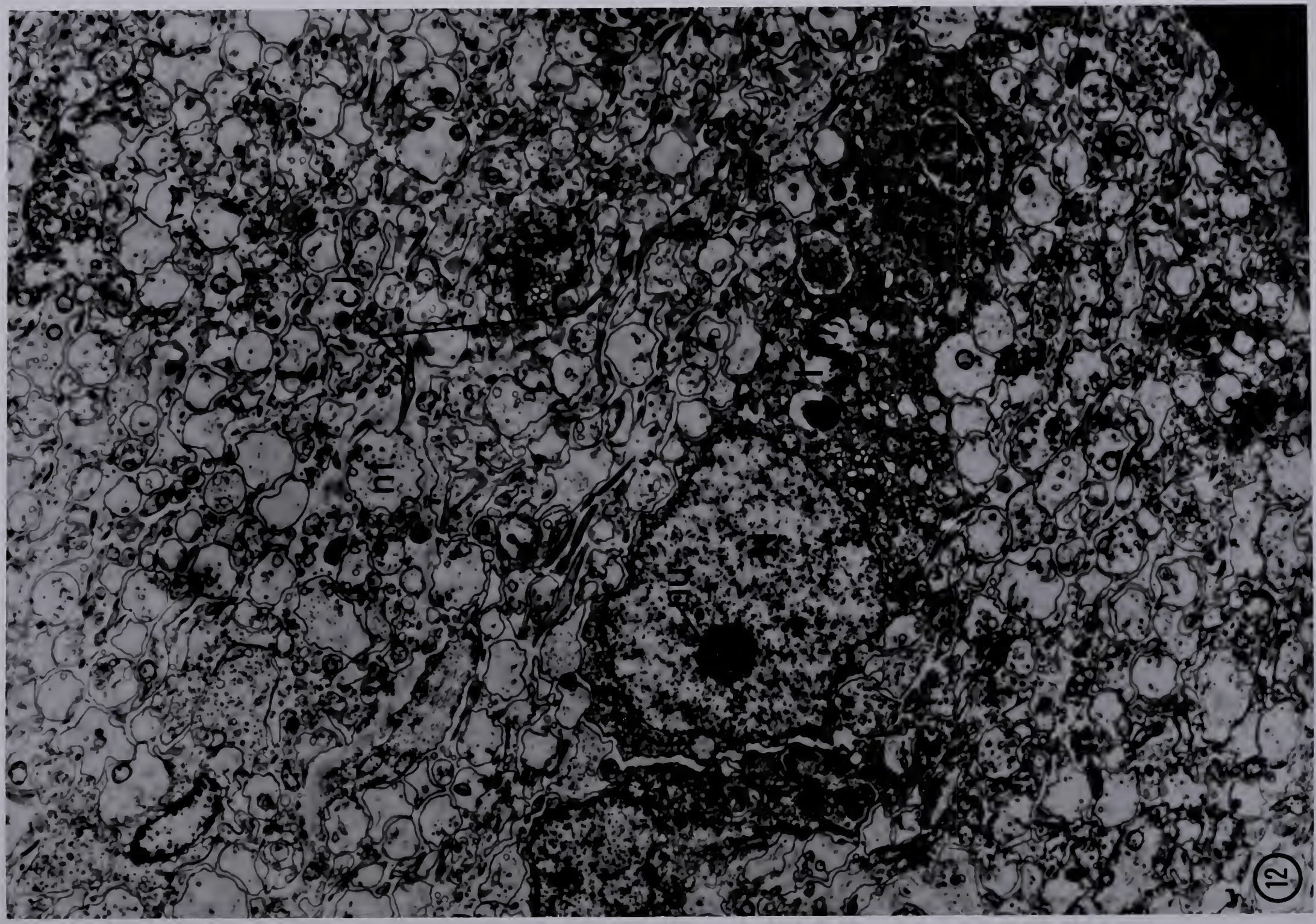
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Fig. 13. Section of the aboral nerve showing the distribution of the collateral nerve fibres.

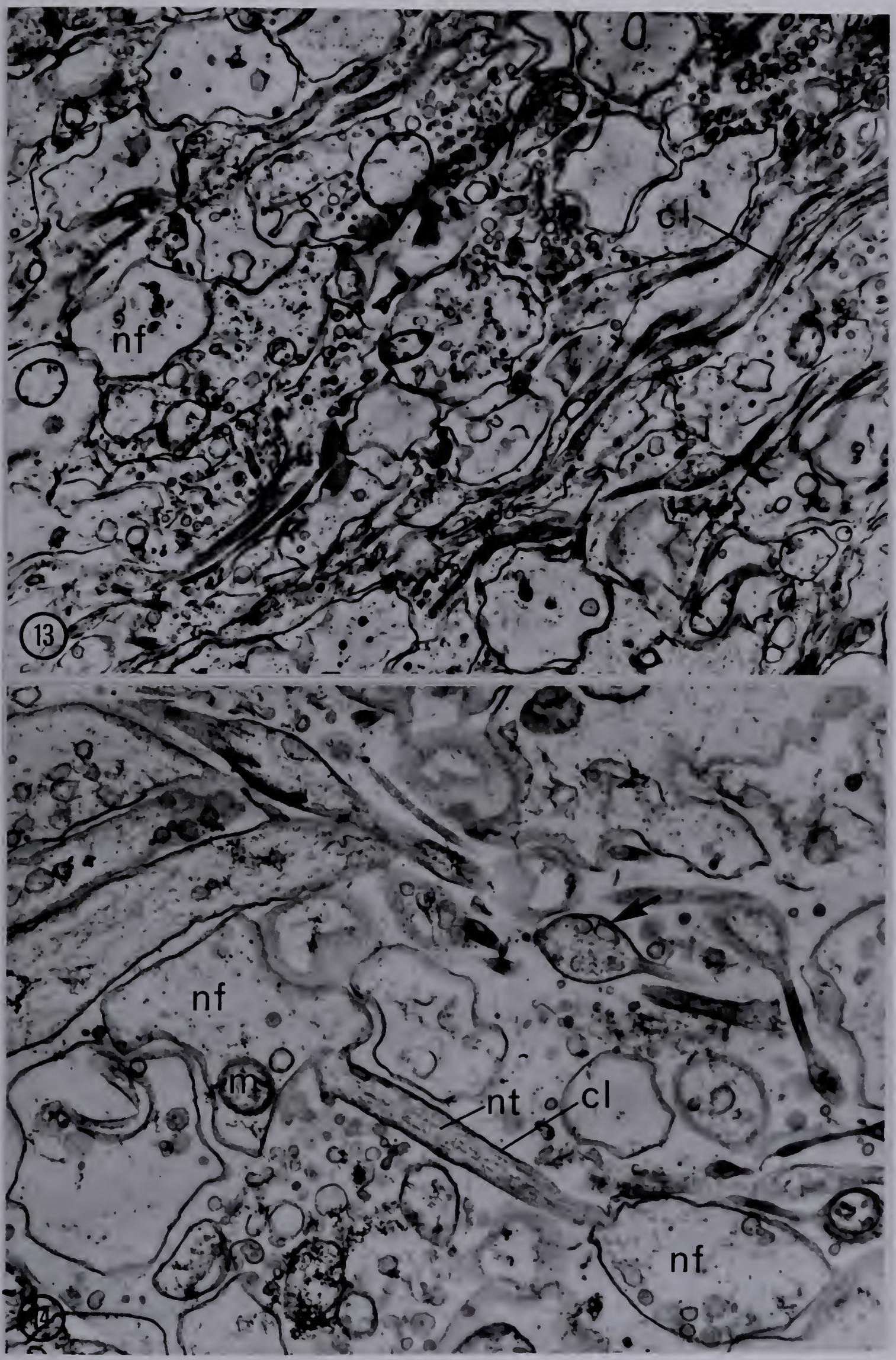
c., collateral fibre; nf, nerve fibre.

Osmium/S-collidine fixation, Epon embedding and lead citrate staining. X 26,500.

Fig. 14. Section of the aboral nerve ring showing what appears to be a collateral fibre joining two adjacent nerve fibres. Swellings, containing vesicles and/or mitochondria can occur in the collateral fibres (arrow).

cl, collateral fibre; nf, nerve fibre; m, mitochondrion; nt, neurotubules.

Glutaraldehyde/osmium fixation, Epon embedding and lead citrate staining. X 27,500.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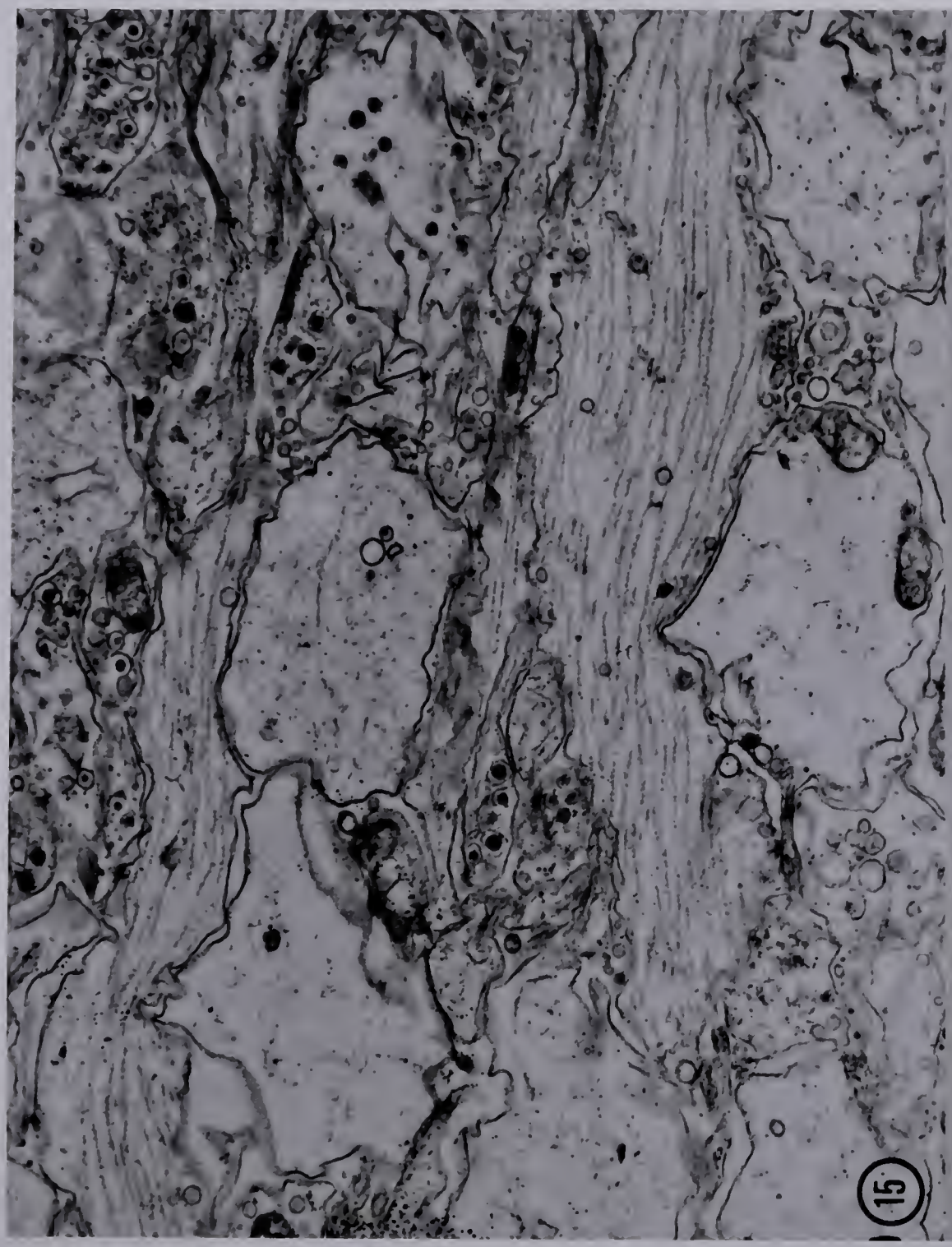
nf
cl
nf
m
nt
cl
nf

Fig. 15. An electron micrograph of the aboral nerve ring showing the presence of neurotubules within the nerve fibres. X 18,200.

Fig. 16. A higher magnification of the neurotubules similar to those shown in Fig. 15. X 59,000.

Glutaraldehyde/osmium fixation, Epon embedding and lead citrate staining.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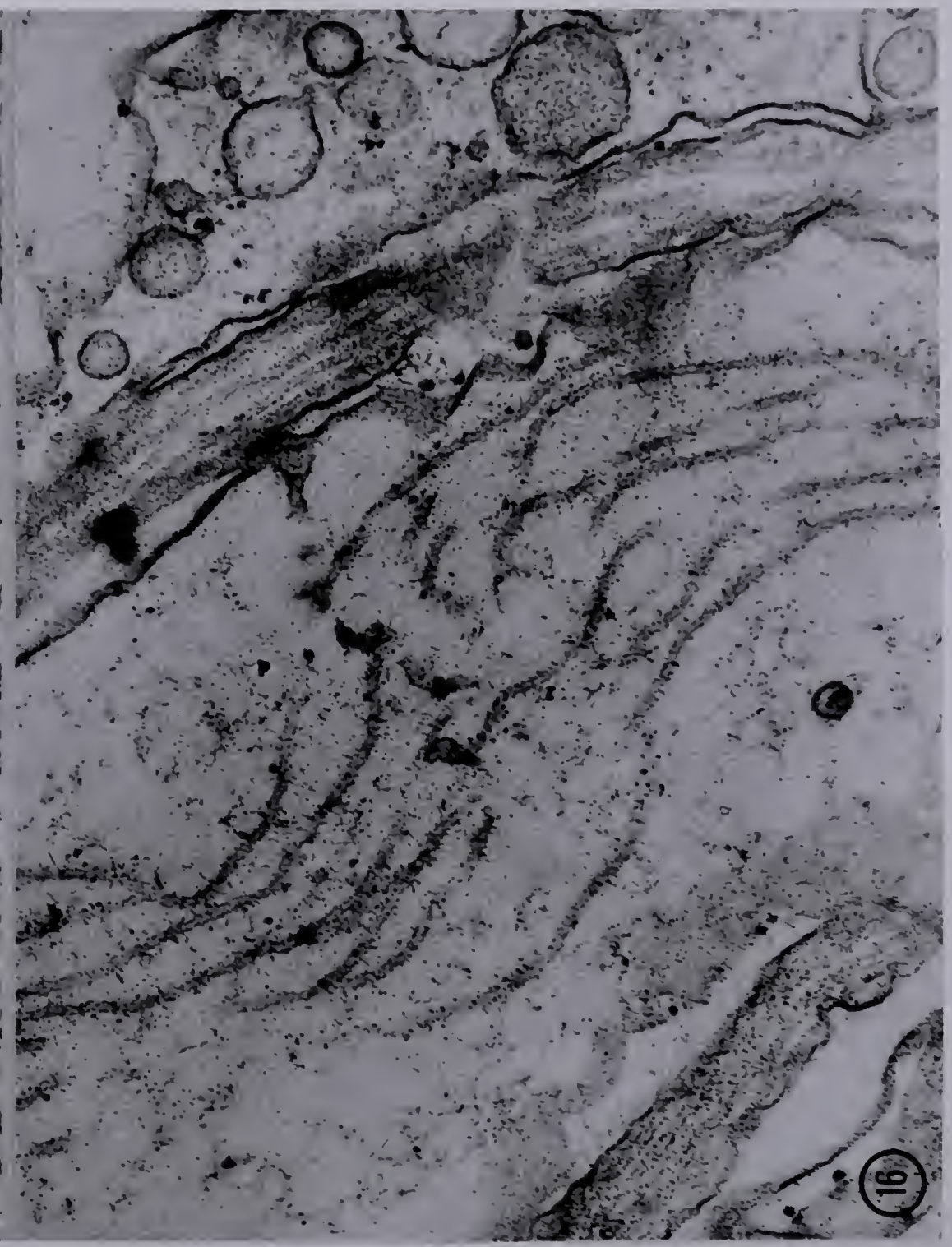

16

Fig. 17. Section of the aboral nerve ring showing nerve fibres containing the two types of vesicles that occur throughout the nervous system. The clear vesicles are presumed to be synaptic vesicles (sn), regularly associated with the synapses and myoneural junctions. The vesicle containing electron dense material is believed to represent neurosecretory vesicles (nsv).

Osmium/S-collidine fixation, Epon embedding and uranyl acetate-lead citrate staining. X 37,500.

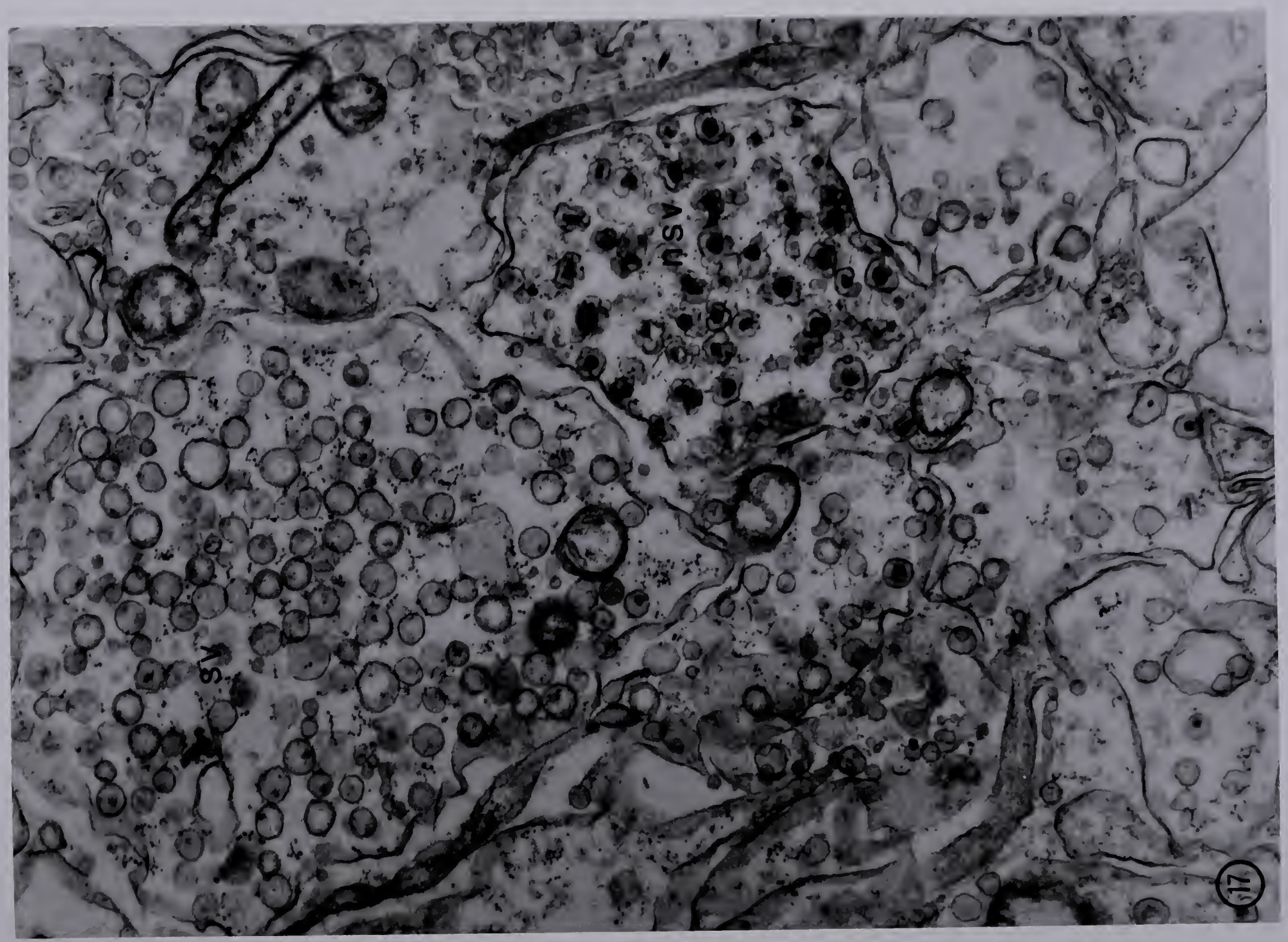

ASV
ASV
17

Fig. 18. An electron micrograph of a presumed two-way type of axo-axonal synapse. Note the diffuse membranes and the lack of a synaptic cleft near the region of contact (arrow). Both described types of vesicles are present together in some fibres but only the clear vesicles are regularly associated with this type ot synapse.

Glutaraldehyde/osmium fixation, Epon embedding and lead citrate staining. X 35,700.

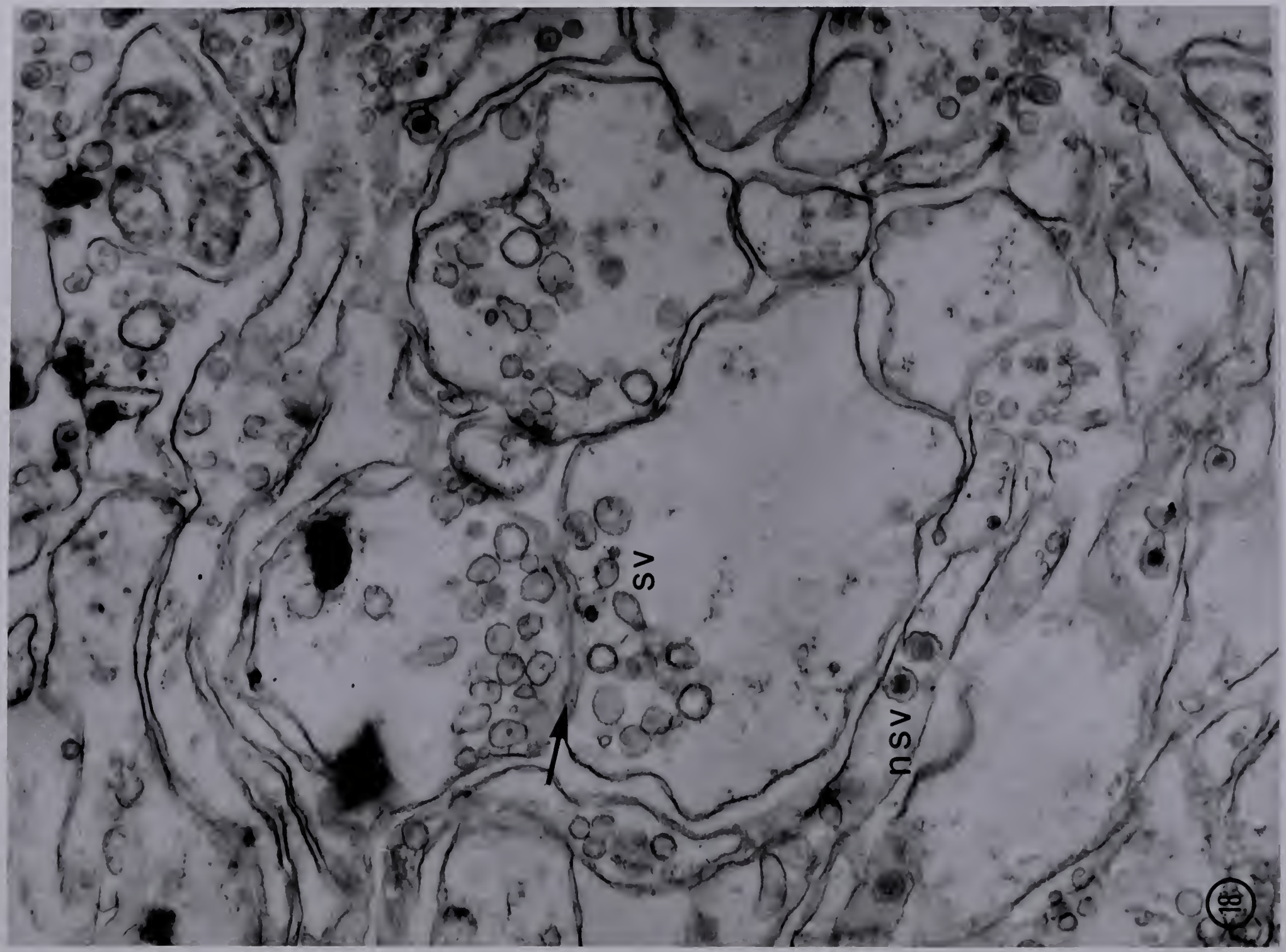

SV
nsv
18

Fig. 19. An electron micrograph showing the fine structure of the non-nervous cells of unknown nature shown in Fig. 6. These cells occur occasionally throughout the nervous system either singly or in large groups.

N, nucleus; og, oval granule; nf, nerve fibre.

Osmium/S-collidine fixation, Epon embedding and uranyl acetate staining. X 12,800.

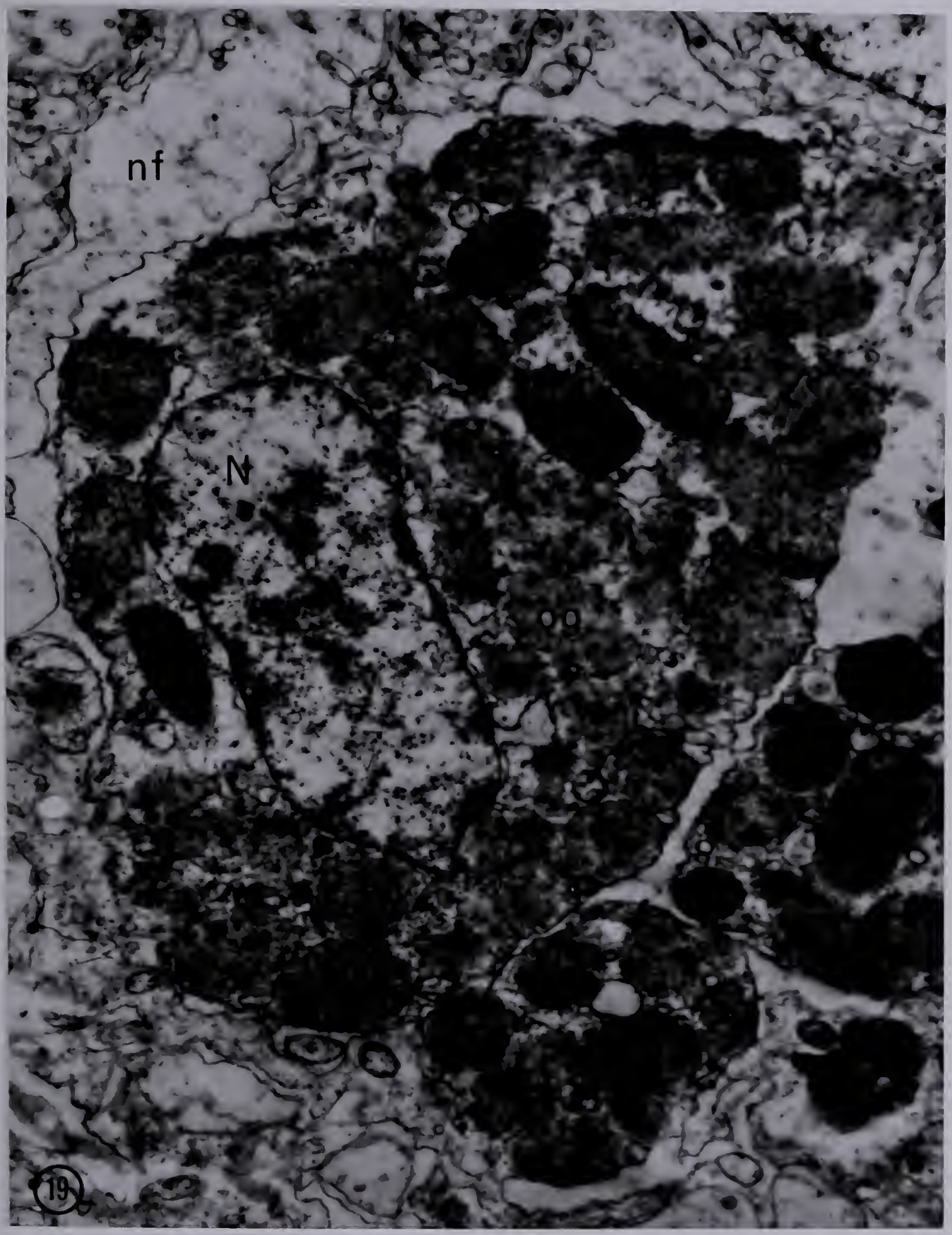

nf
N
19

Fig. 20. Section showing the strands of collagen fibres that occasionally occur within the nervous system. These collagen fibres appear to be the only supportive tissue within the nervous system.

c, collagen fibre; nf, nerve fibre; nt, neurotubules; f, neurofilaments.

Glutaraldehyde/osmium fixation, Epon embedding and lead citrate staining. X 46,000.

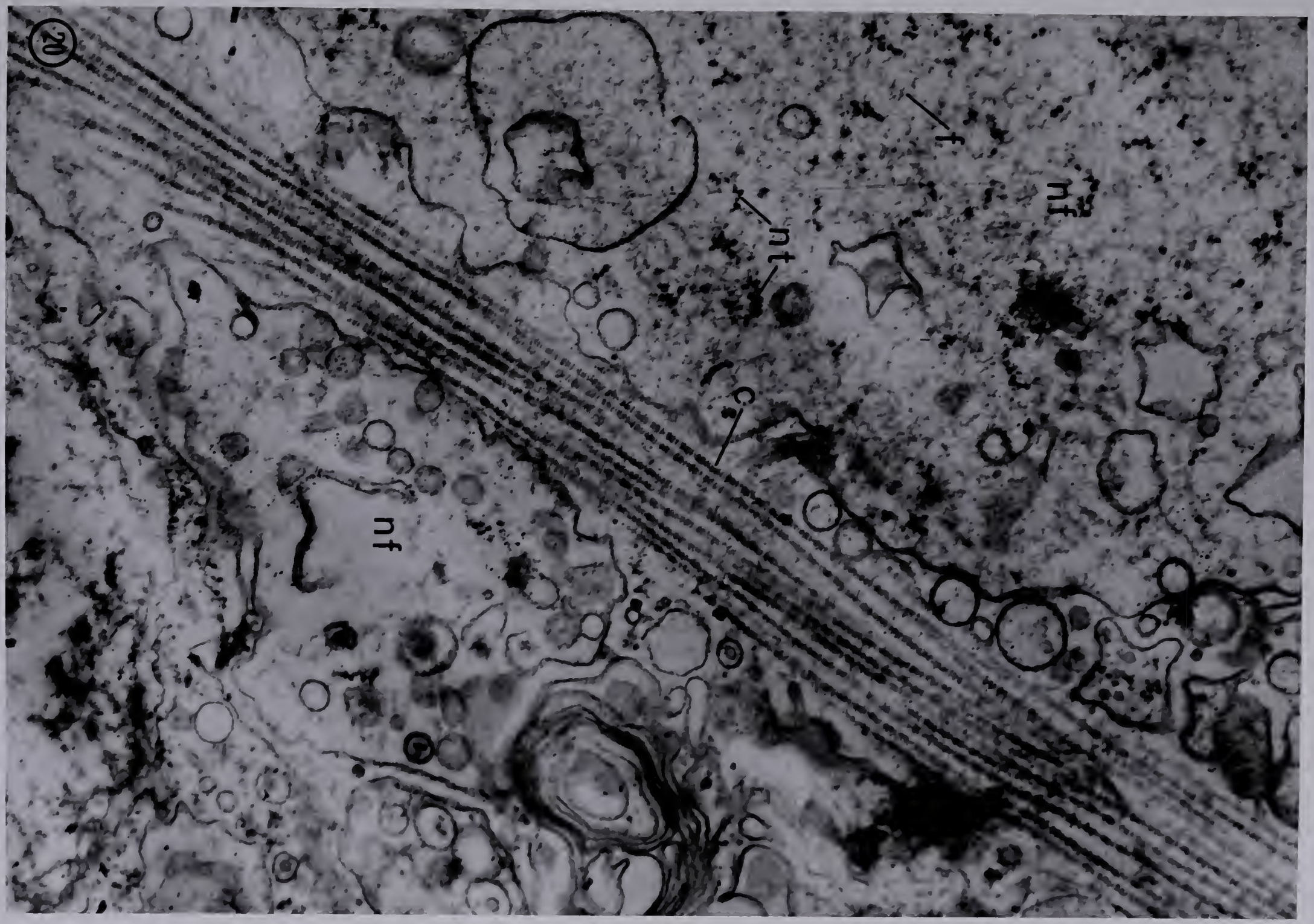

f
nf
nt
ci
nf

Fig. 21. Electron micrograph of a nerve cell with many of the structures commonly found in the large bi-, and multipolar nerve cells. Note the apparent continuity of the endoplasmic reticulum with the nuclear envelope (arrow).

N, nucleus; G, Golgi complex and associated vesicles; ER, rough endoplasmic reticulum; ne, nuclear envelope; r, rosettes of ribosomes (polyribosomes); mv, multivesicular body; m, mitochondrion; lp, lipofuscin granule.

Osmium/S-collidine fixation, Epon embedding and uranyl acetate-lead citrate staining. X 20,000.

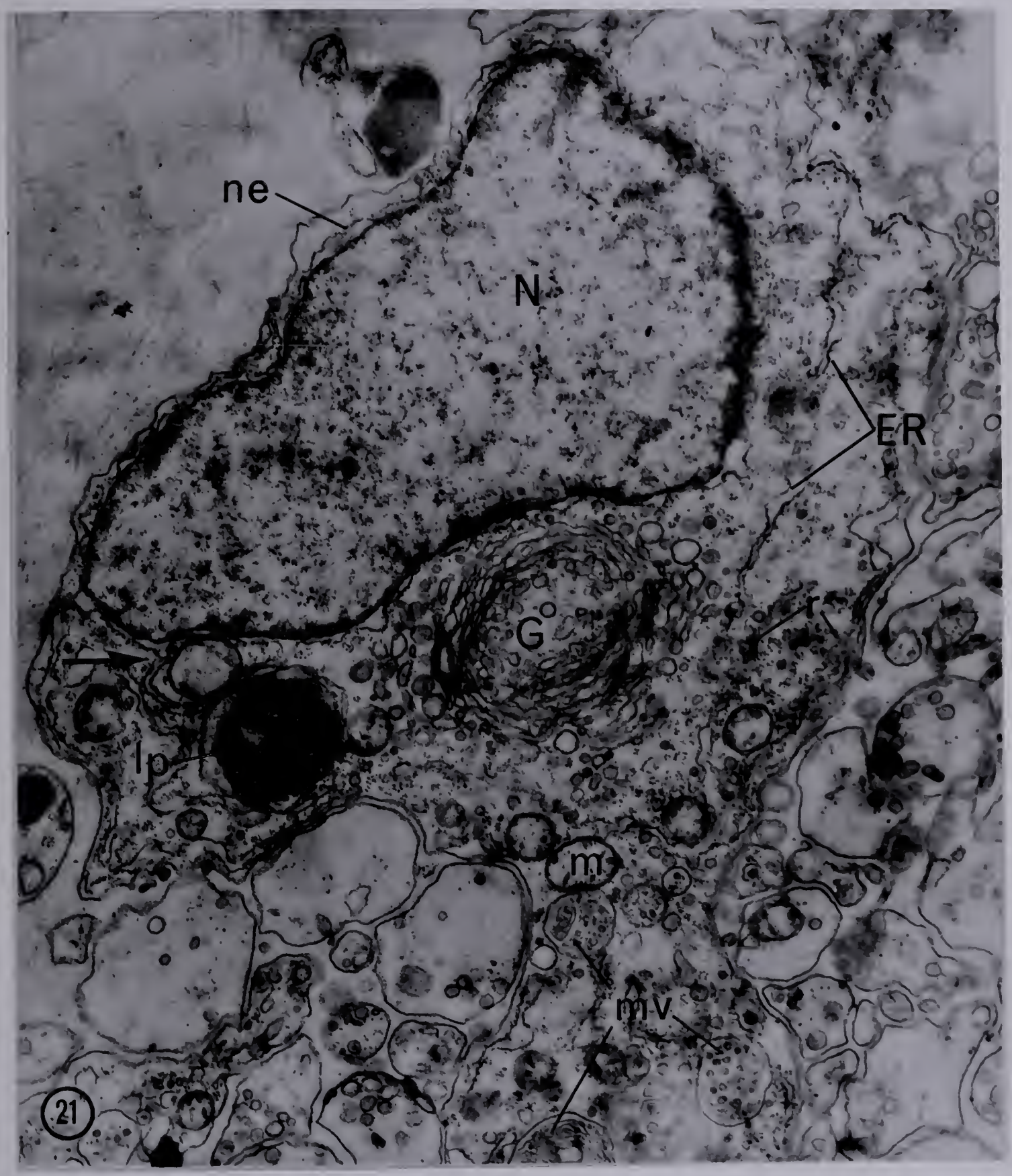

ne
N
ER
G
lp
m
mv
21

Fig. 22. A portion of a nerve cell body showing the various structures commonly found in the cytoplasm.

N, nucleus; G, Golgi complex; ER, rough endoplasmic reticulum; m, mitochondrion; r, ribosomes; lb, lamellate body containing a highly osmophilic substance (see Figs. 23, 24 and 25).

Osmium/S-collidine fixation, Epon embedding and uranyl acetate-lead citrate staining. X 38,500.

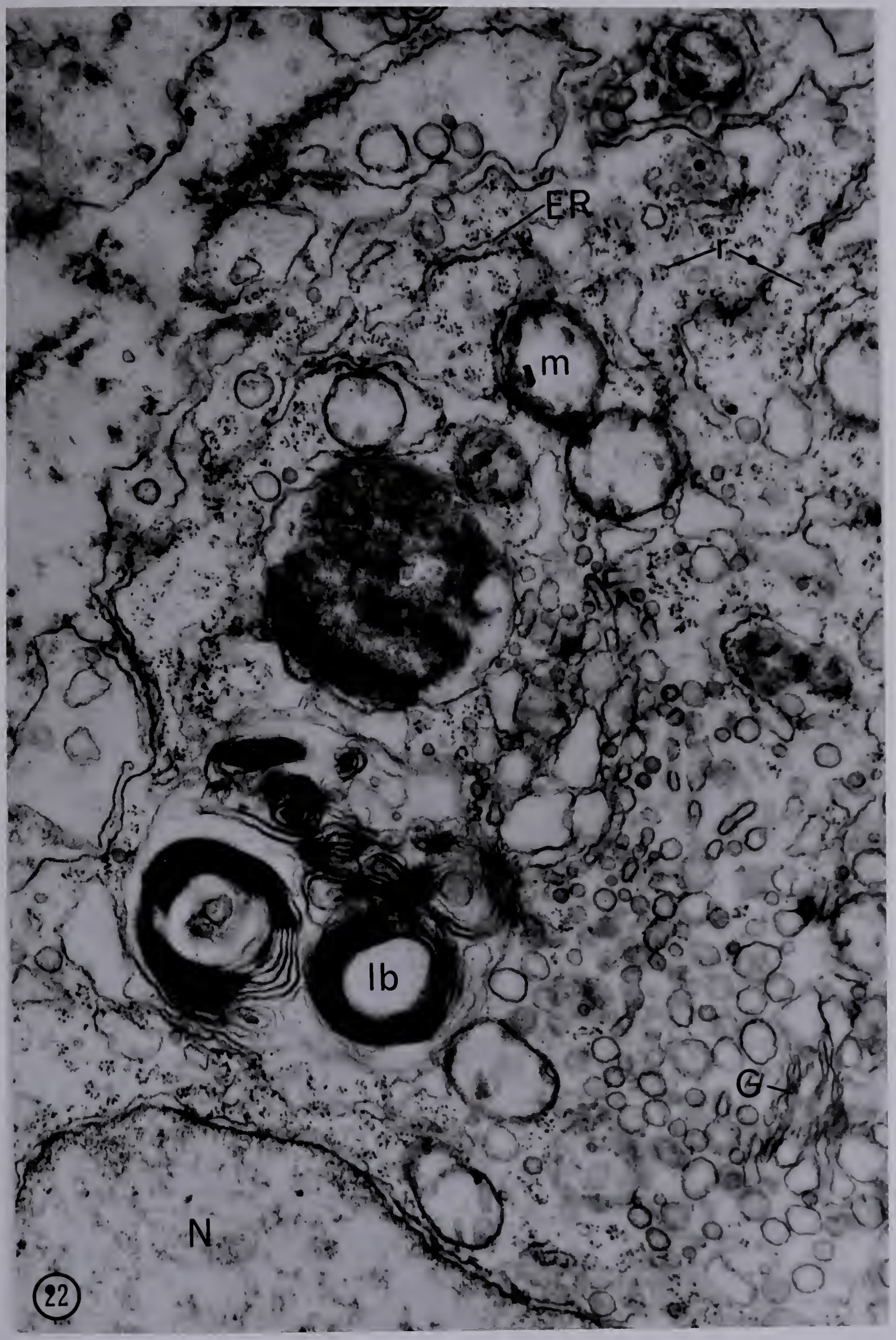

ER
r
m
lb
G
N
22

Fig. 23. Section of a nerve cell containing lamellate bodies. These structures are thought to be a type of lysosome involved in the formation of lipofuscin pigment.

N, nucleus; lb, lamellate body; nf, nerve fibre.

Osmium/S-collidine fixation, Epon embedding and lead citrate staining. X 12,000.

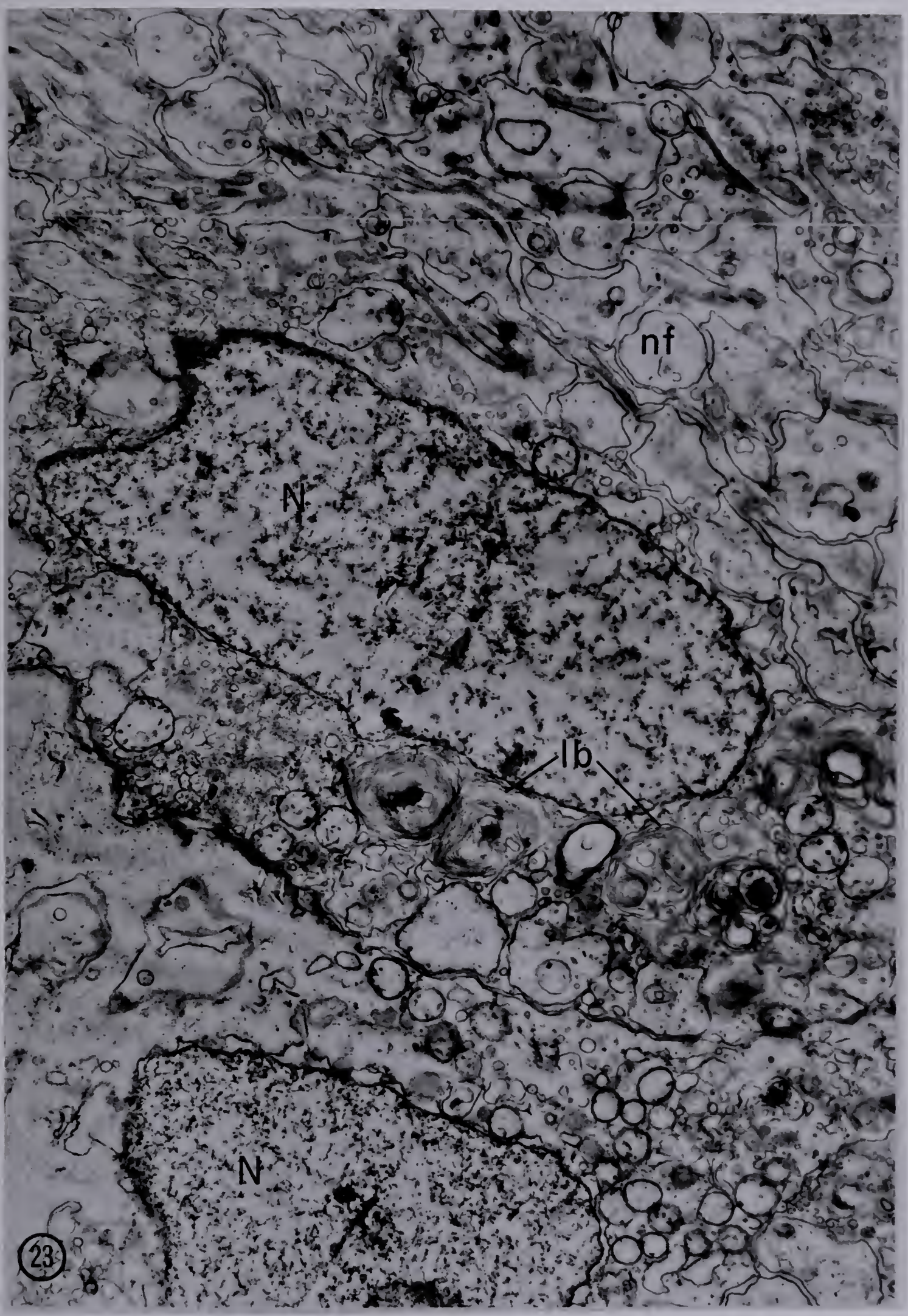

nf
N
lb
N
23

Fig. 24. A higher magnification of the cytoplasm of a nerve cell containing lamellate bodies similar to those shown in Fig. 23. Several multivesicular bodies (arrows) can also be seen in the cytoplasm.

Osmium/S-collidine fixation, Epon embedding and lead citrate staining. X 22,000.

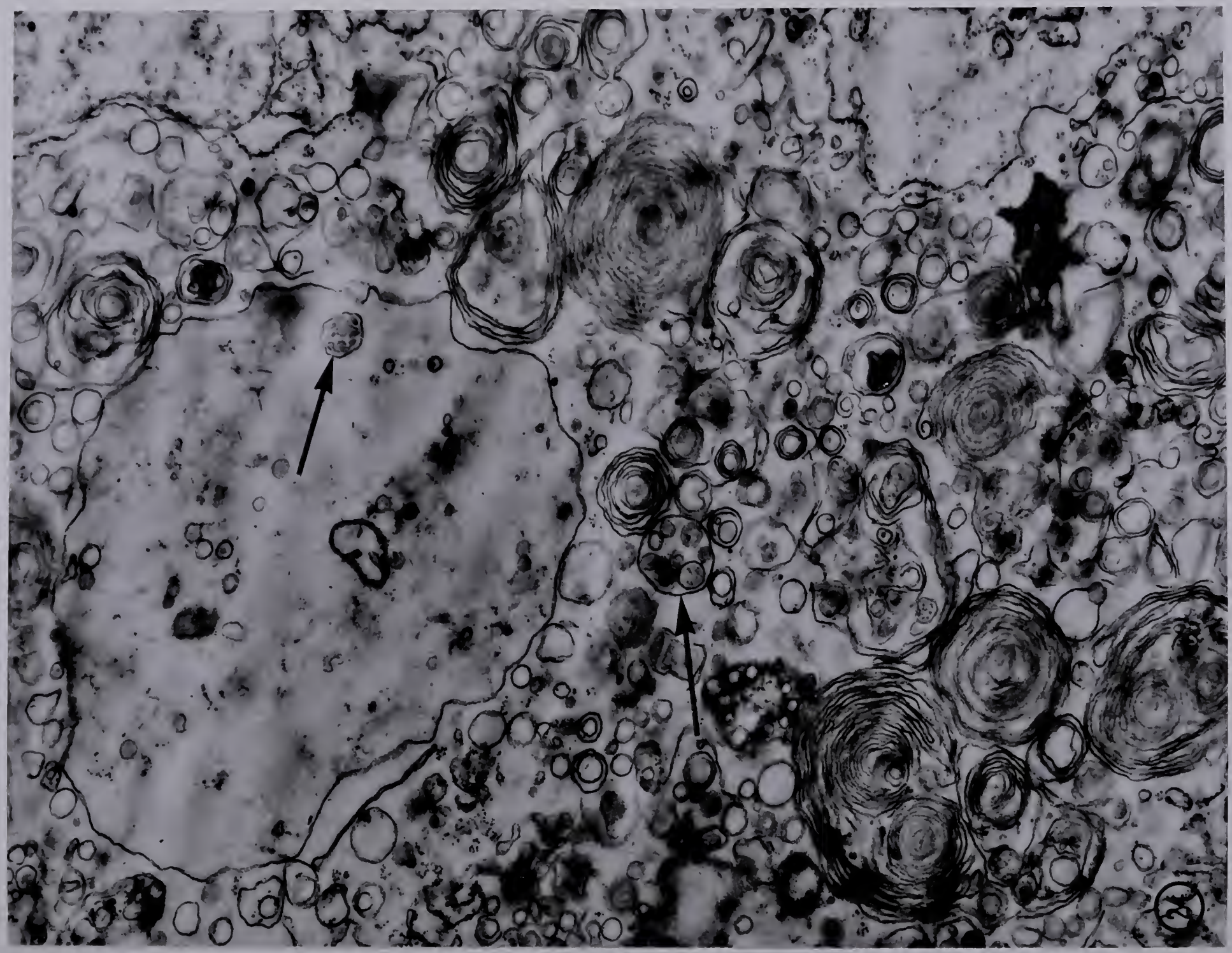


Fig. 25. Section of a nerve cell depicting the formation of the lipfuscin pigment. The pigment formation appears to begin in the lamellate body (lb). Elaboration of the pigment takes place between the membranes (b) until the membraneous structure of the lamellate body is totally obscured, forming large granules (g) within the cell body. Also see Fig. 26.

Osmium/S-collidine fixation, Epon embedding and lead citrate staining. X 16,200.

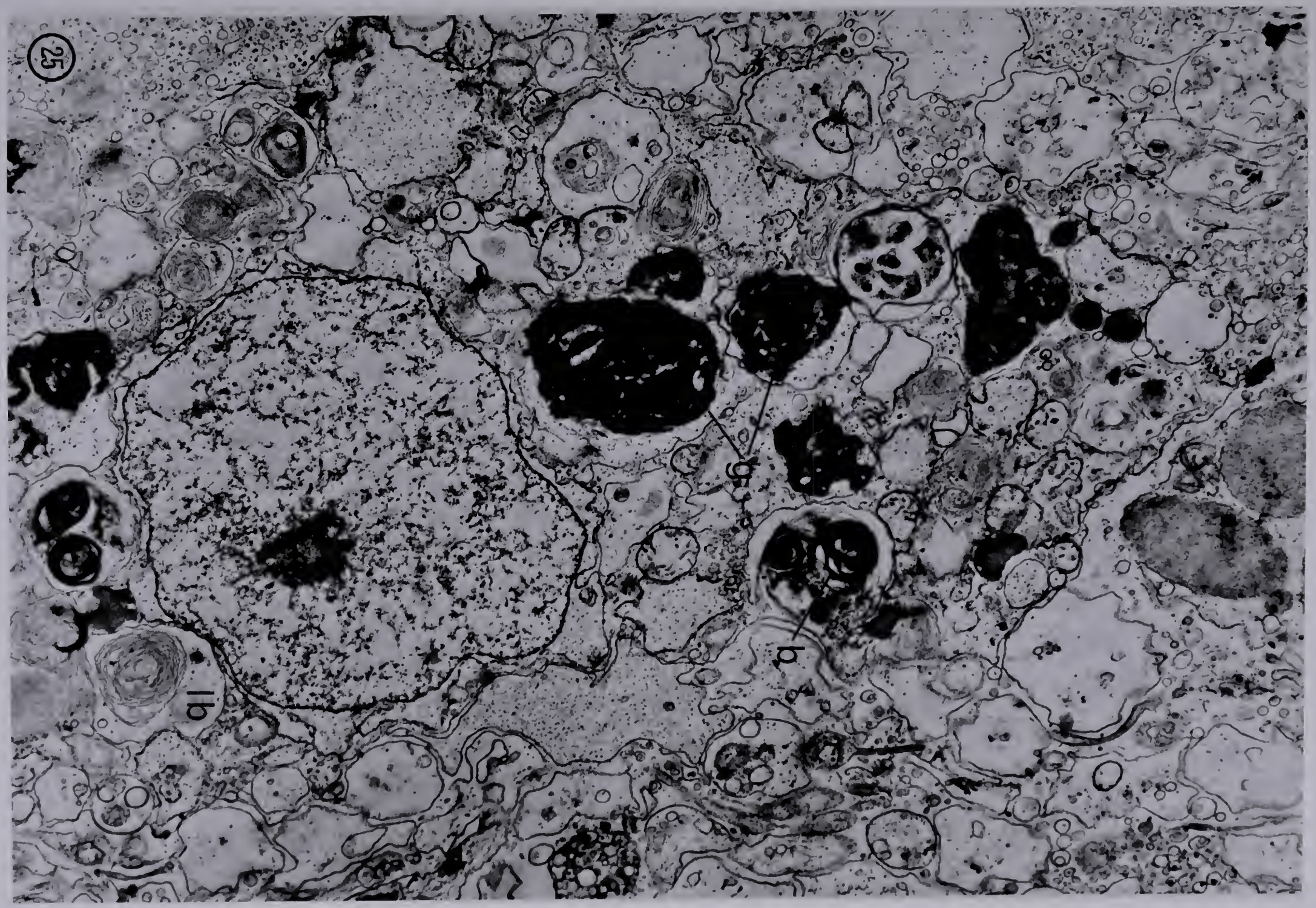

25
g
b
a1
b

Fig. 26. Section of a nerve cell containing several large lipofuscin granules similar to those shown in Fig. 25.

Osmium/S-collidine fixation, Epon embedding and lead citrate staining. X 12,800.

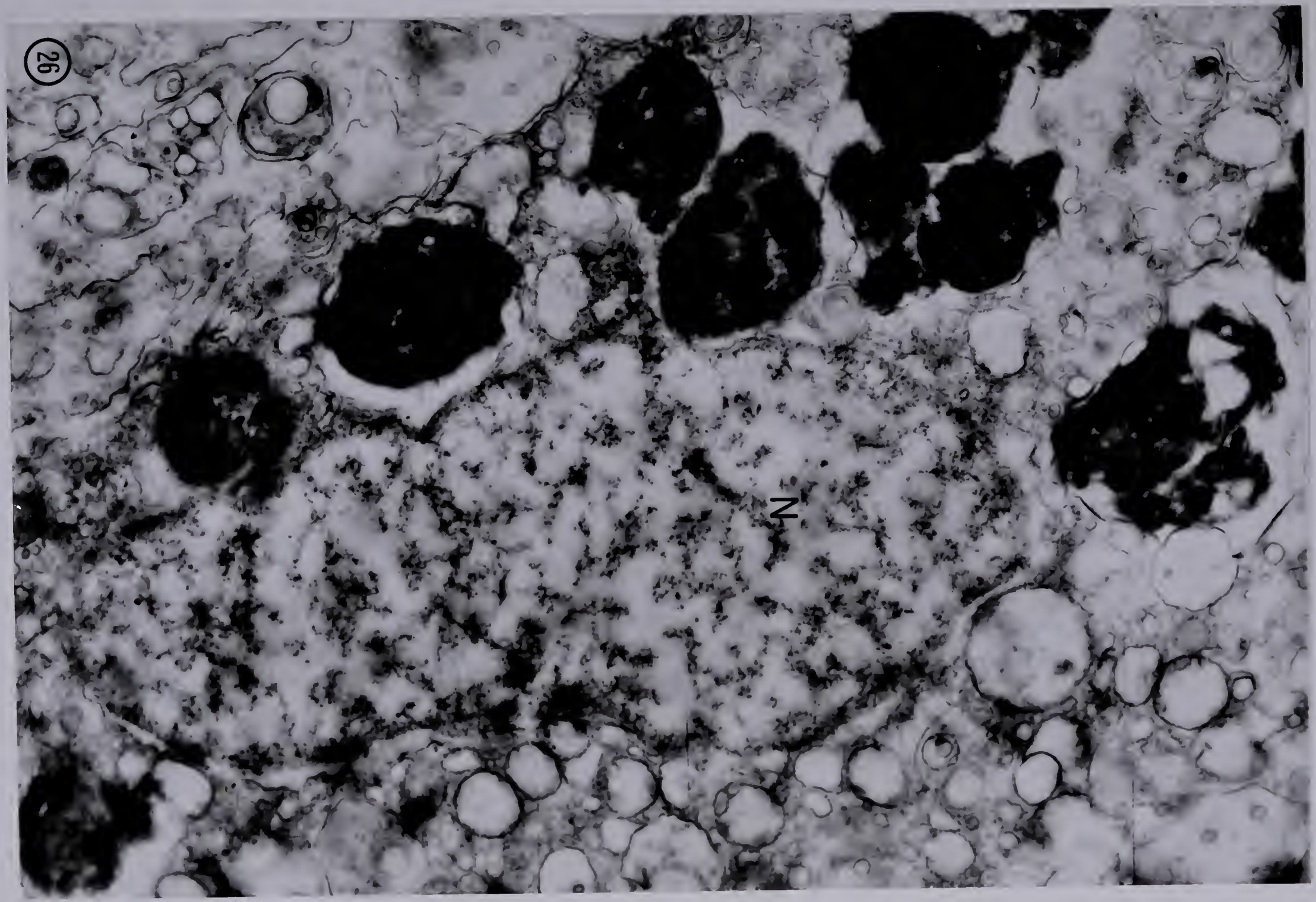

26
N

Fig. 27. Section of a nerve cell containing numerous lipofuscin granules. Note the pycnotic appearance of the nucleus suggesting possible degeneration of the nerve cell.

N, pycnotic nucleus; L, lipofuscin granule; lb, lamellate body. X 6,700.

Fig. 28. An electron micrograph of several nerve cells in the advanced stages of pigment formation. This condition is generally found in the older animals. X 6,000.

Osmium/S-collidine fixation, Epon embedding and uranyl acetate-lead citrate staining.

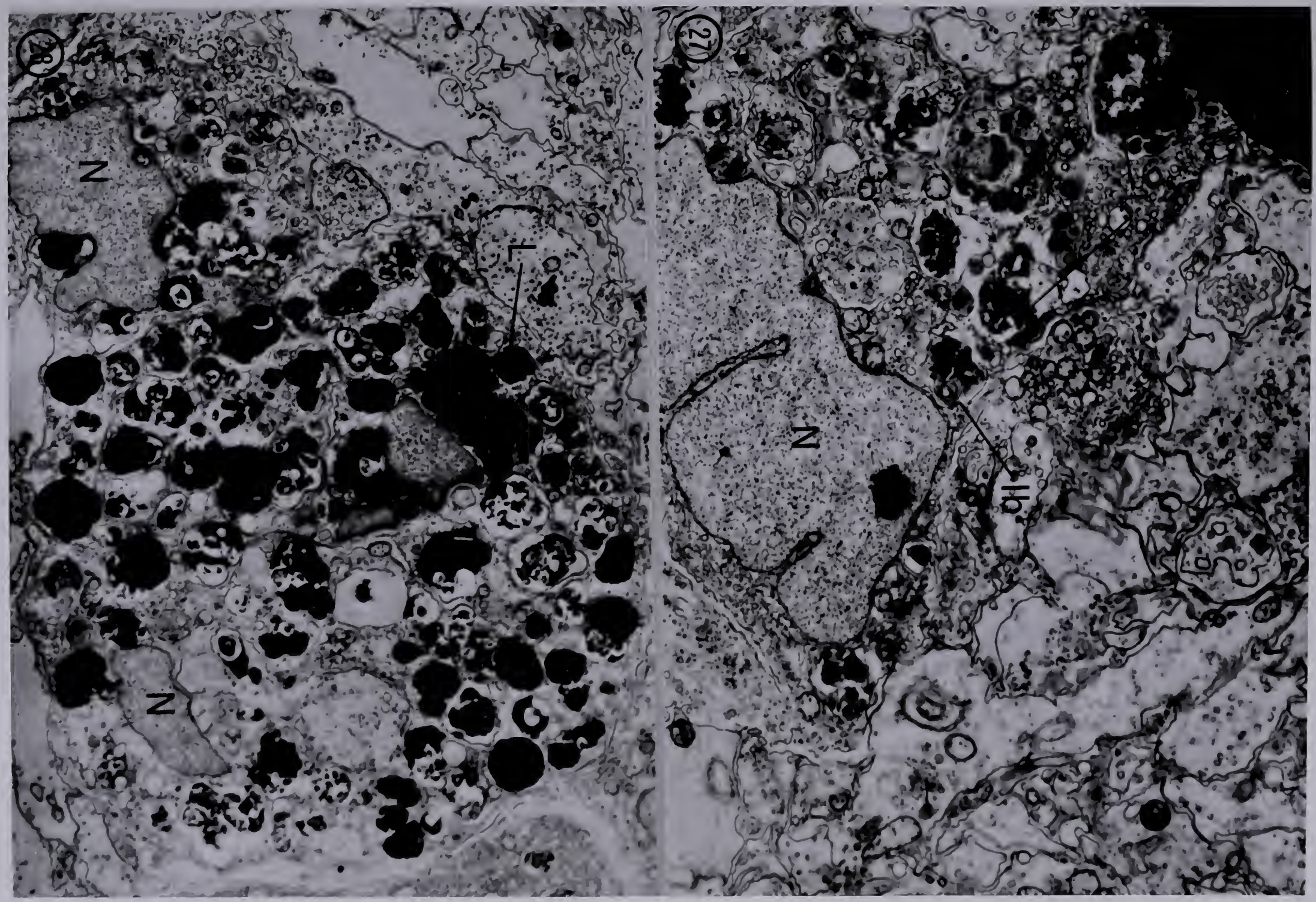

N
N
N
lb

Fig. 29. Section of the transverse commissure and chiasma located in the axillary brachials at the base of the arm pairs.

tc, transverse commissure; ch, chiasma; n, nerve trunk coming from the aboral nerve ring; bn, brachial nerve cord. X 725.

Fig. 30. A light micrograph of a cross section of the arm.

bn, brachial nerve cord; fm, flexor muscle; fg, ciliated food groove. X 490.

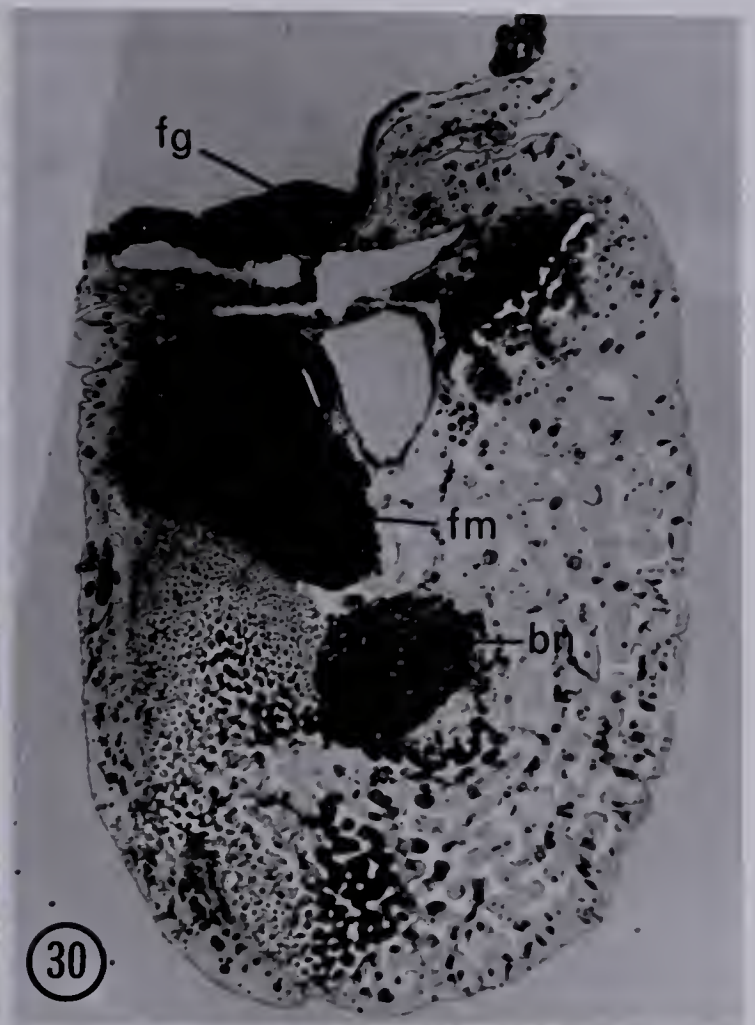
Fig. 31. A longitudinal section of the brachial nerve cord showing the relationship of the nerve cell bodies to the neuropile.

cr, cell rind; np, neuropile; p, central core of parallel fibres en route; sp, spicule. X 1,600.

Fig. 32. A light micrograph of the tract of large nerve fibres passing around the cortex of the nerve ring. These large nerve fibres continue out along the brachial nerve cords. X 720.

Osmium/S-collidine fixation, Epon embedding and Richardson's staining.

29



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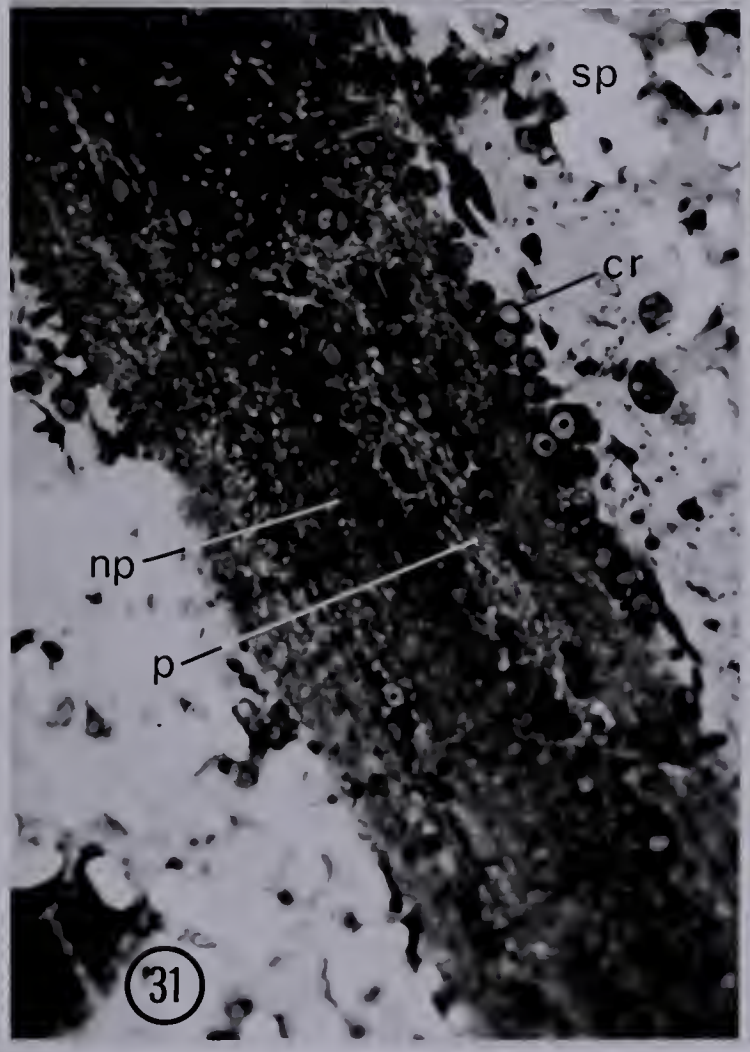
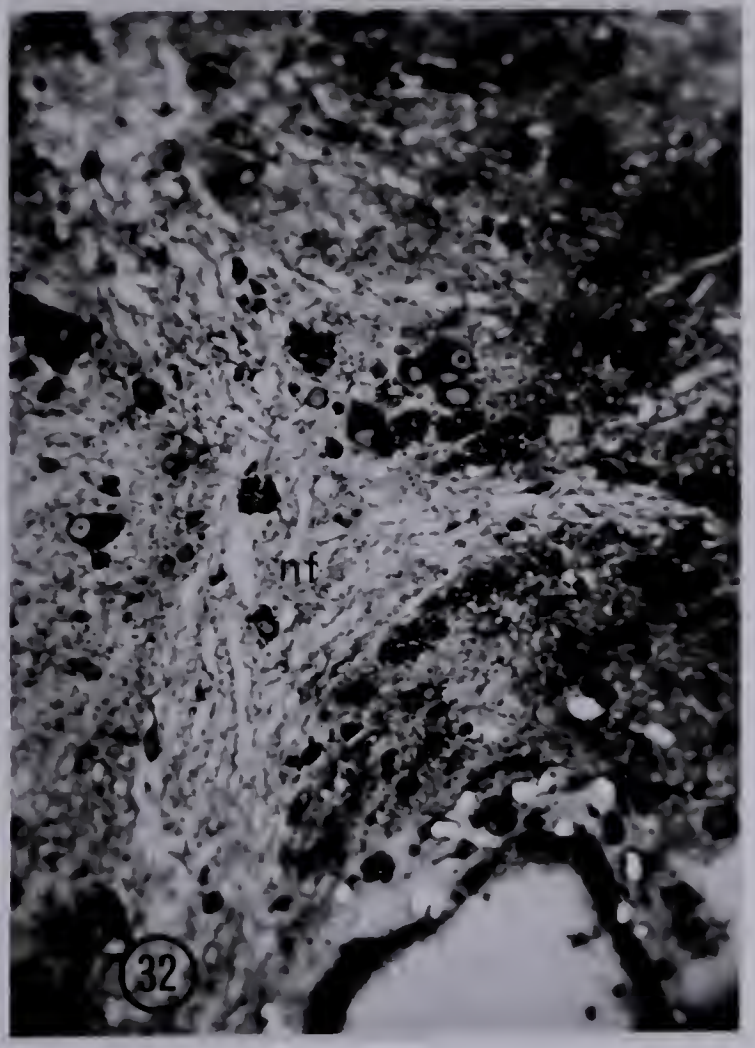


31



32

Fig. 33. Section of a portion of a nerve cell in the brachial nerve cord. Note lipofuscin granules are also present in the cell bodies of the nerve cord.

N, nucleus of the nerve cell; L, lipofuscin granule; nsv, neurosecretory vesicles.

Glutaraldehyde/osmium fixation, Epon embedding and uranyl acetate-lead citrate staining. X 21,000.

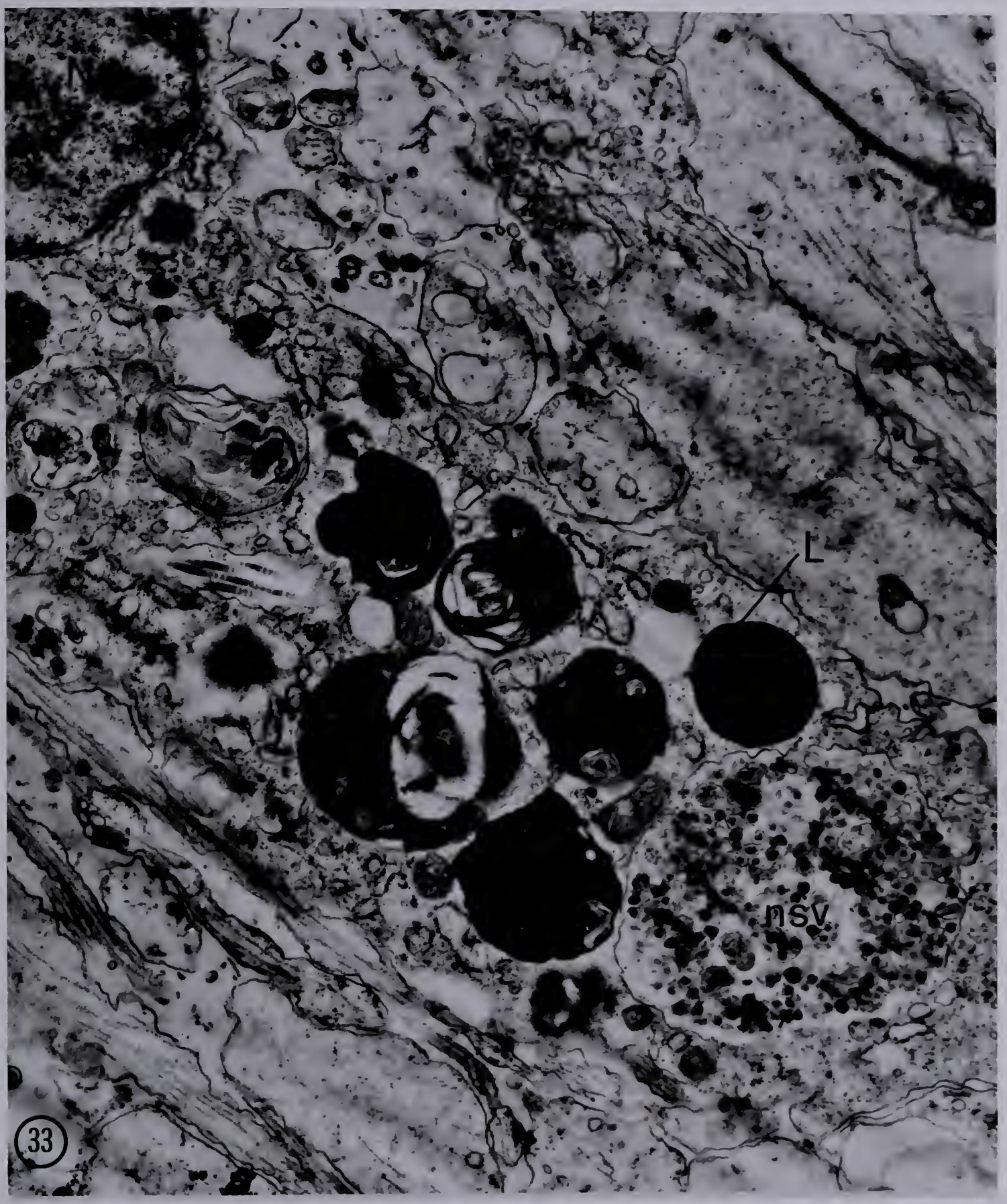

N
Pa
L
nsv
33

Fig. 34. A cross section of the large nerve fibres found in the central core of the brachial nerves. Nerve fibres appearing near the nucleus (N) have diameters typically found in areas of the nervous system outside this central core.

nf, large nerve fibre; nsv, neurosecretory vesicle.

Osmium/S-collidine fixation, Epon embedding and uranyl acetate-lead citrate staining. X 10,800.

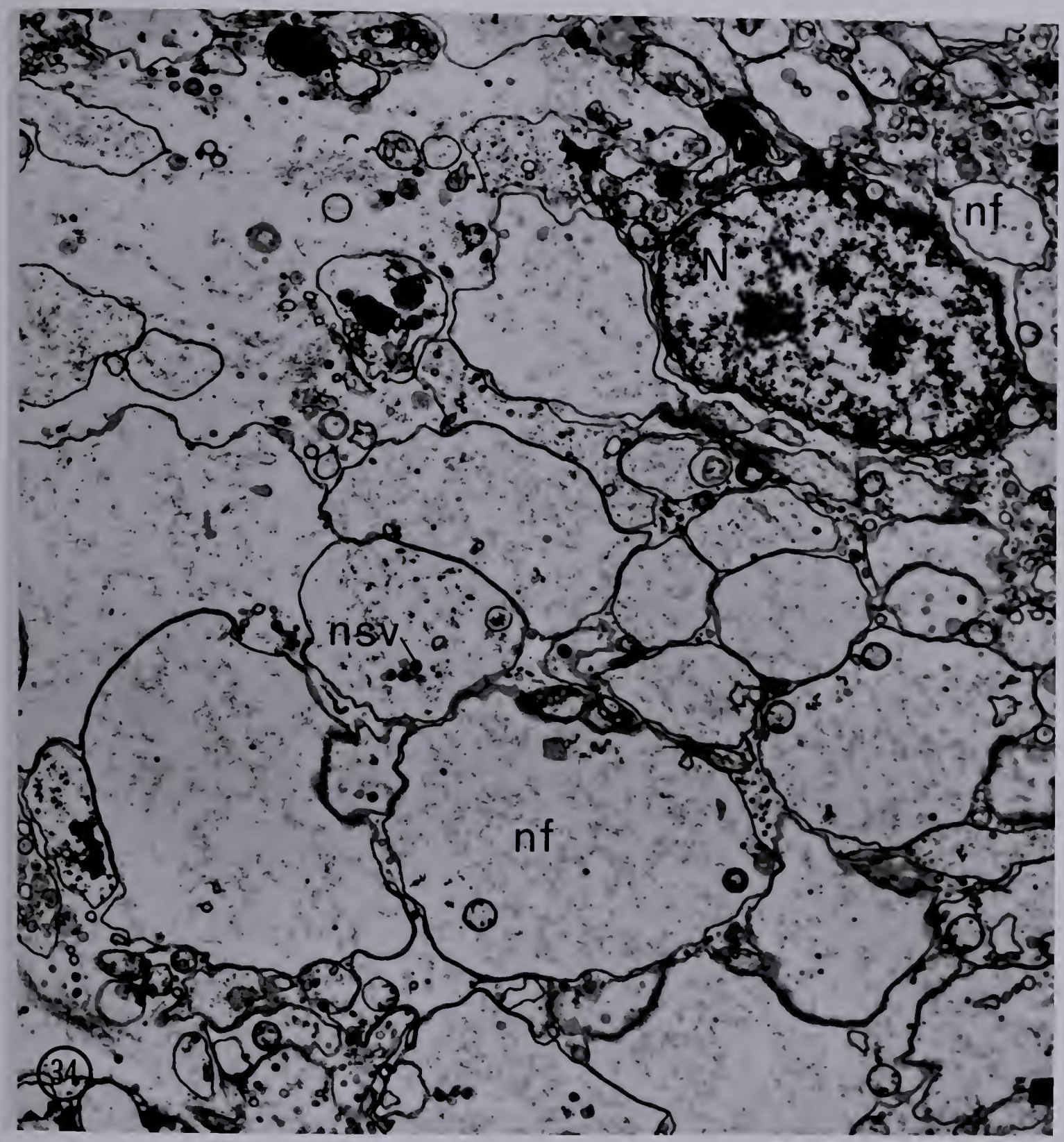

nf
N
nsv
nf

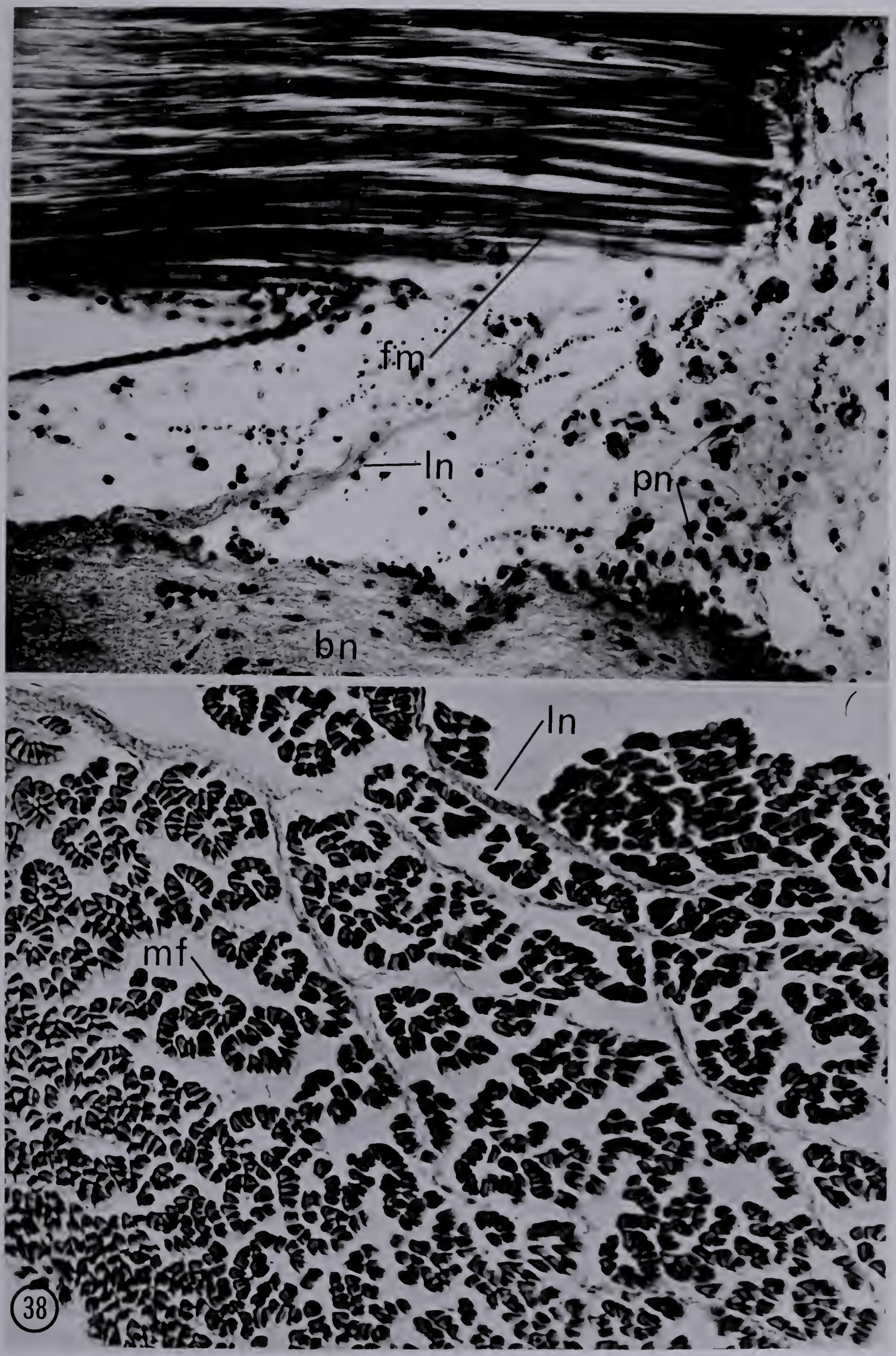
Fig. 37. A longitudinal section of the arm showing the lateral nerve arising from the brachial nerve cord to innervate the flexor muscle.

bn, brachial nerve; fm, flexor muscle; ln, lateral nerve; pn, portion of nerve branch to the pinnules. X 1,200.

Fig. 38. A cross section of the flexor muscle showing the penetration of the nerves into the flexor muscle. The nerve fibres anastomose throughout the entire width of the flexor muscles. The appearance of the muscle structure has been slightly distorted from the paraffin embedding treatment.

mf, muscle fibre; ln, lateral nerve penetrating the flexor muscle. X 1,800.

Susa fixation, paraffin embedding and Masson's trichrome staining.



38

Fig. 39. Longitudinal section showing the fine structure of the flexor muscle. The muscle fibres are indistinctly striated with a wavy Z-line (Z). Note the prominent sarcoplasmic reticulum near the nucleus of the muscle cell (N) with additional membrane systems of undetermined function penetrating the muscle fibres (arrow).

SR, sarcoplasmic reticulum; t, triglyceride droplets; m, mitochondrion; sl, sarcolemma; mf, muscle fibre.

Osmium/S-collidine fixation, Epon embedding and uranyl acetate-lead citrate staining. X 6,500.

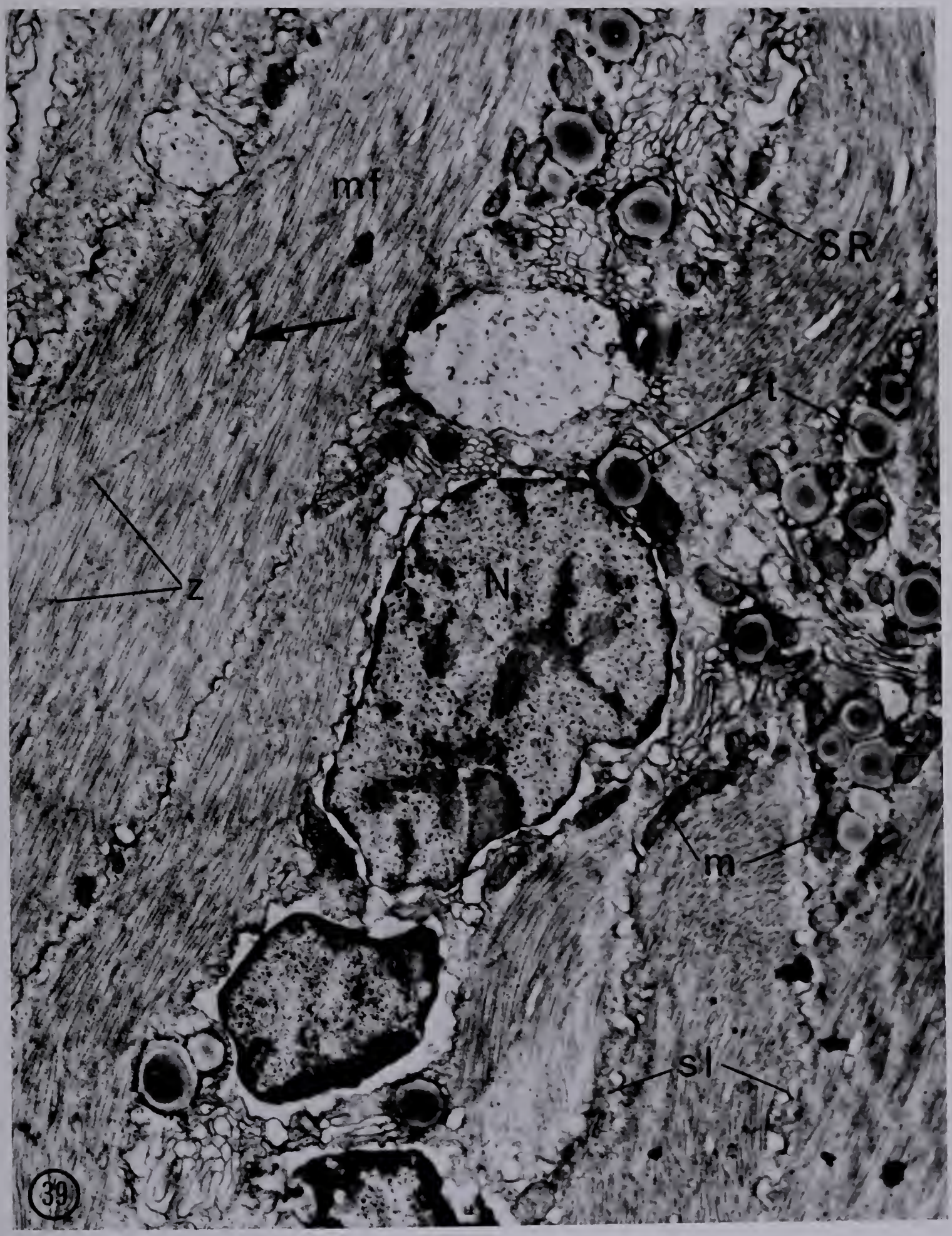

mf
SR
t
Z
N
m
sl
39

Fig. 40. An electron micrograph of two nerve fibres (nf) passing between the fibres of the flexor muscle. The nerve fibres pass at right angles to the longitudinal plane of the muscle fibres (mf).

Osmium/S-collidine fixation, Epon embedding and uranyl acetate-lead citrate staining. X 21,000.

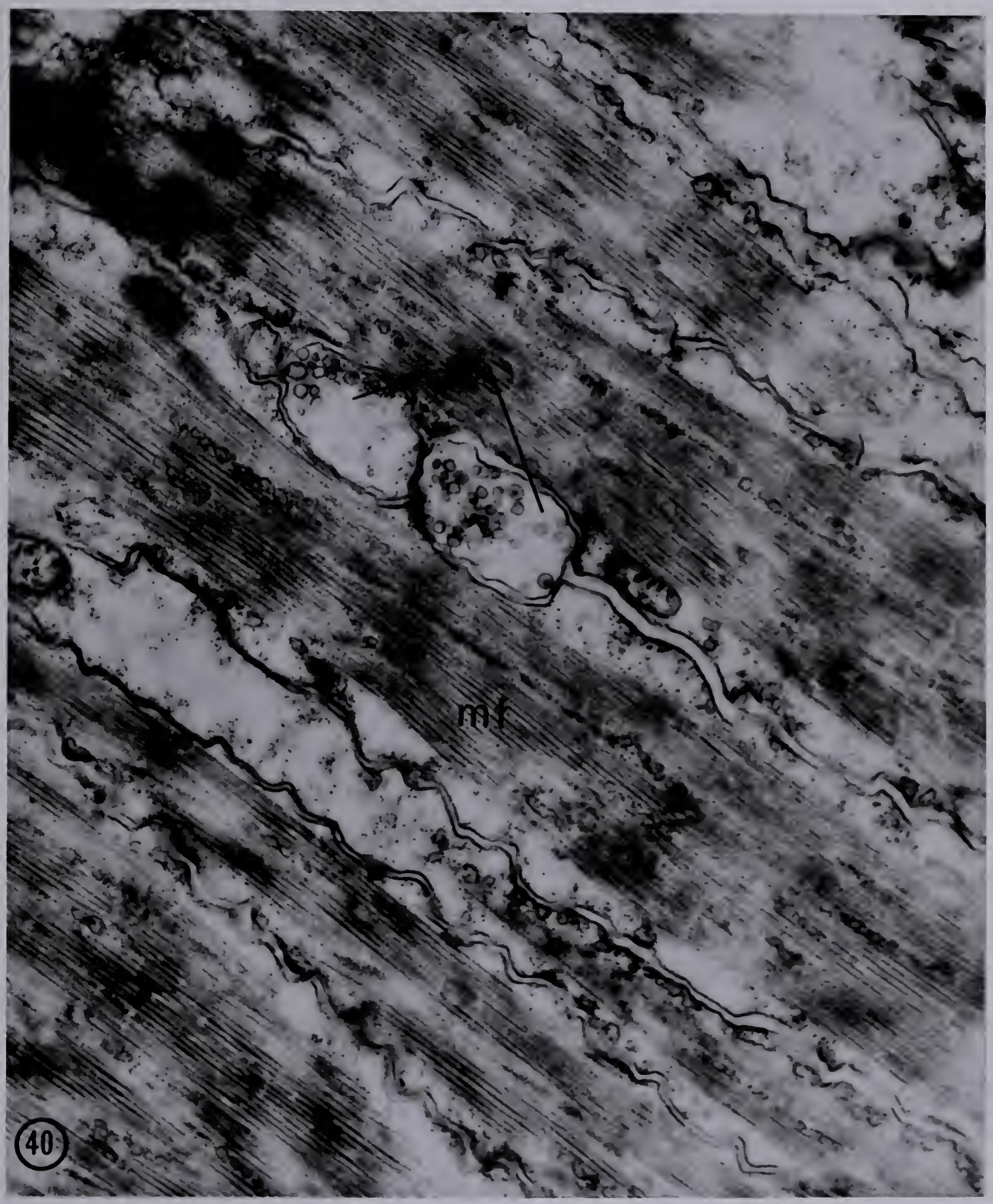
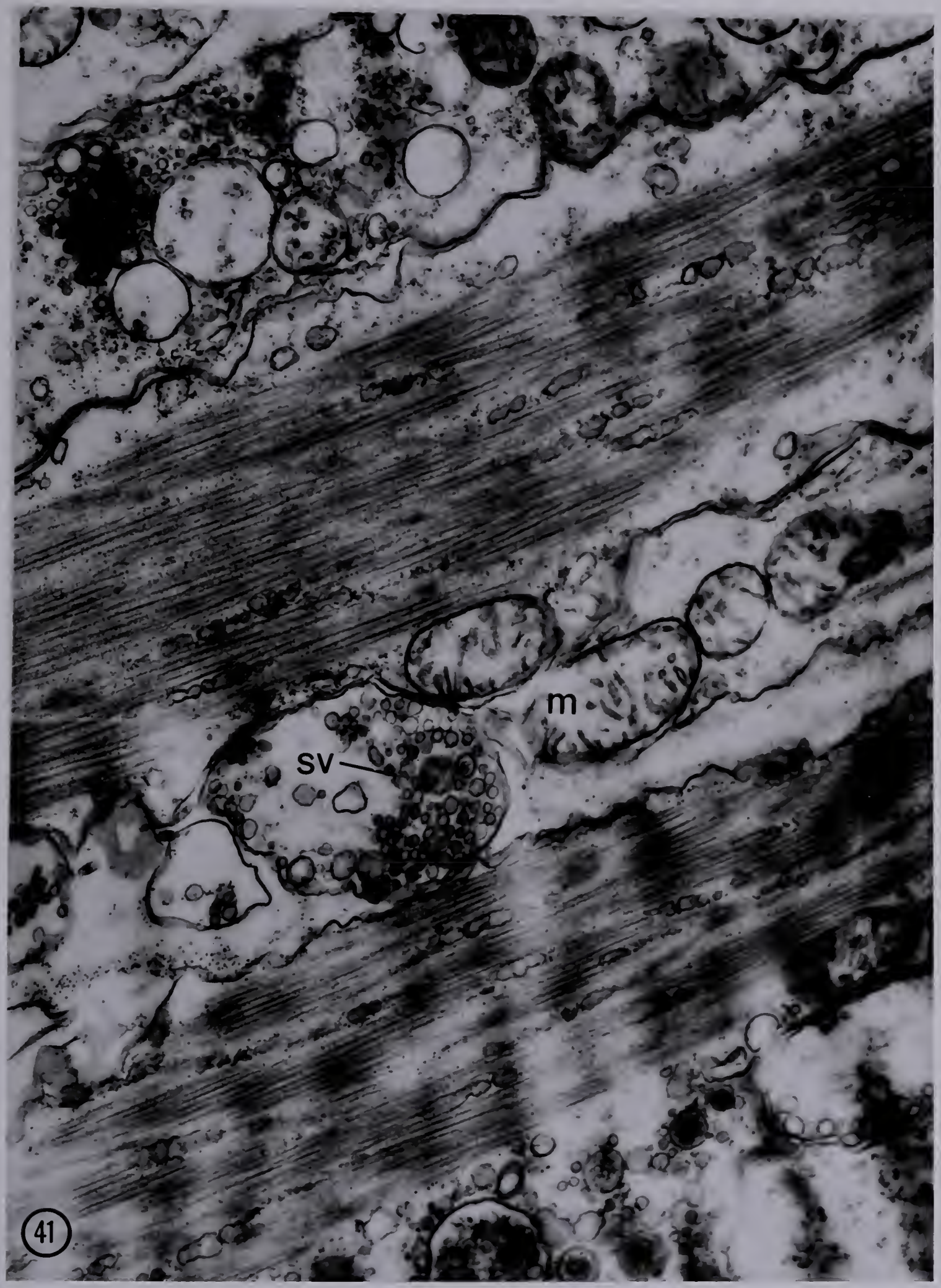

mf
40

Fig. 41. Electron micrograph of a neuromuscular junction. The presence of synaptic vesicles (sv) and muscle mitochondria (m) beneath the sarcolemma has been taken to represent a neuromuscular junction.

Osmium/S-collidine fixation, Epon embedding and uranyl acetate-lead citrate staining. X 24,000.


m
sv
41

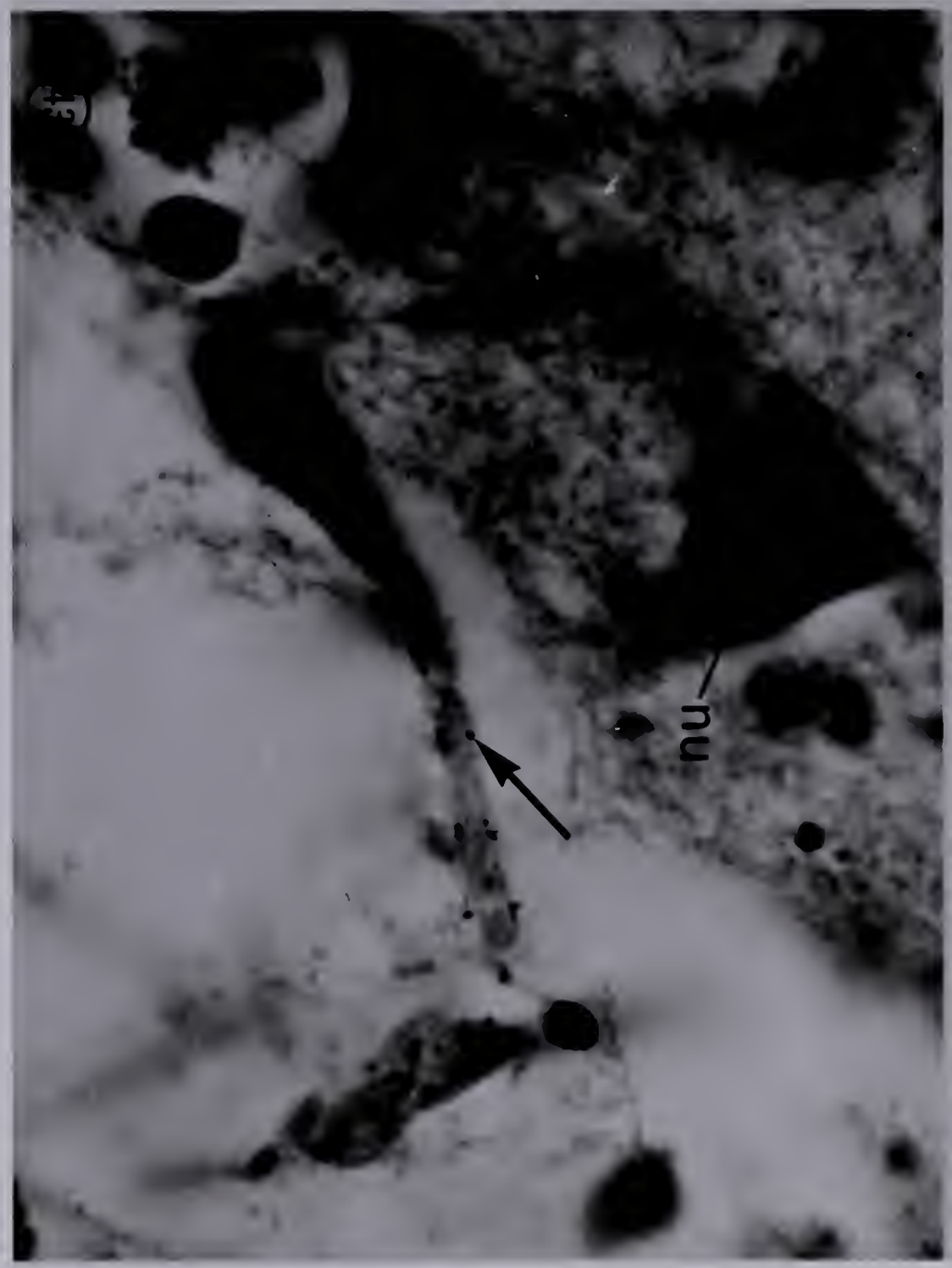
Figs. 42 and 43. Light micrographs of the large bi-, and multipolar nerve cells containing Gomori positive inclusions within the cell body and out along the cell processes (arrow). These nerve cells have been regarded as neurosecretory cells.

N, nucleus; nu, nucleolus.

Susa fixation, paraffin embedding and paraldehyde fuchsin staining.

Fig. 42. X 5,000.

Fig. 43. X 4,700.


43
nu

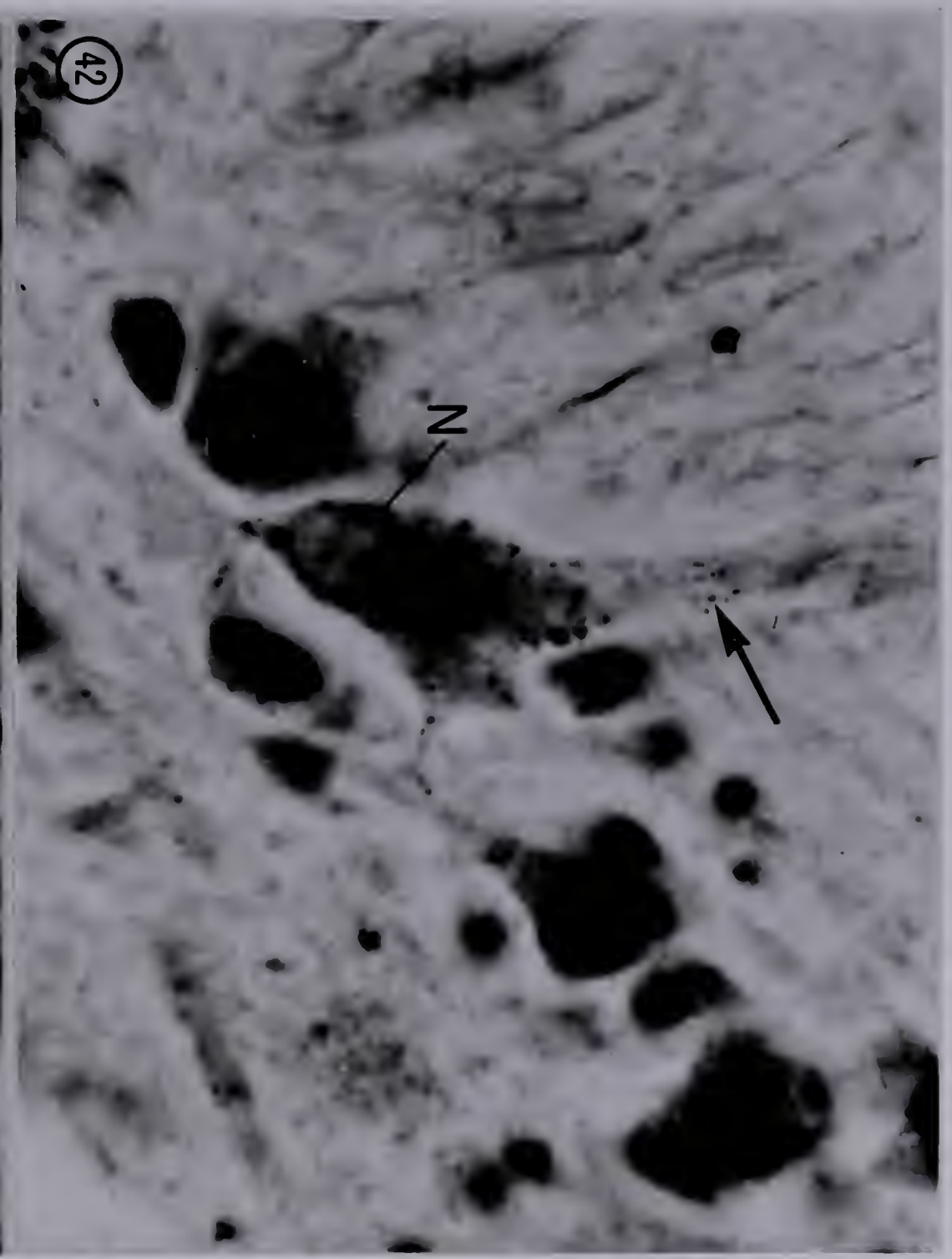

42
N

Fig. 44. An electron micrograph of a presumed neurosecretory cell shown in Figs. 42 and 43. Note the numerous neurosecretory vesicles (nsv) occurring within the cytoplasm of the cell body and out along the cell processes (cp). The nucleolus which is generally associated with the neurosecretory cells is not shown in this section.

N, nucleus; nf, nerve fibre; nt, neurotubules.

Glutaraldehyde/osmium fixation, Epon embedding and uranyl acetate-lead citrate staining. X 17,600.

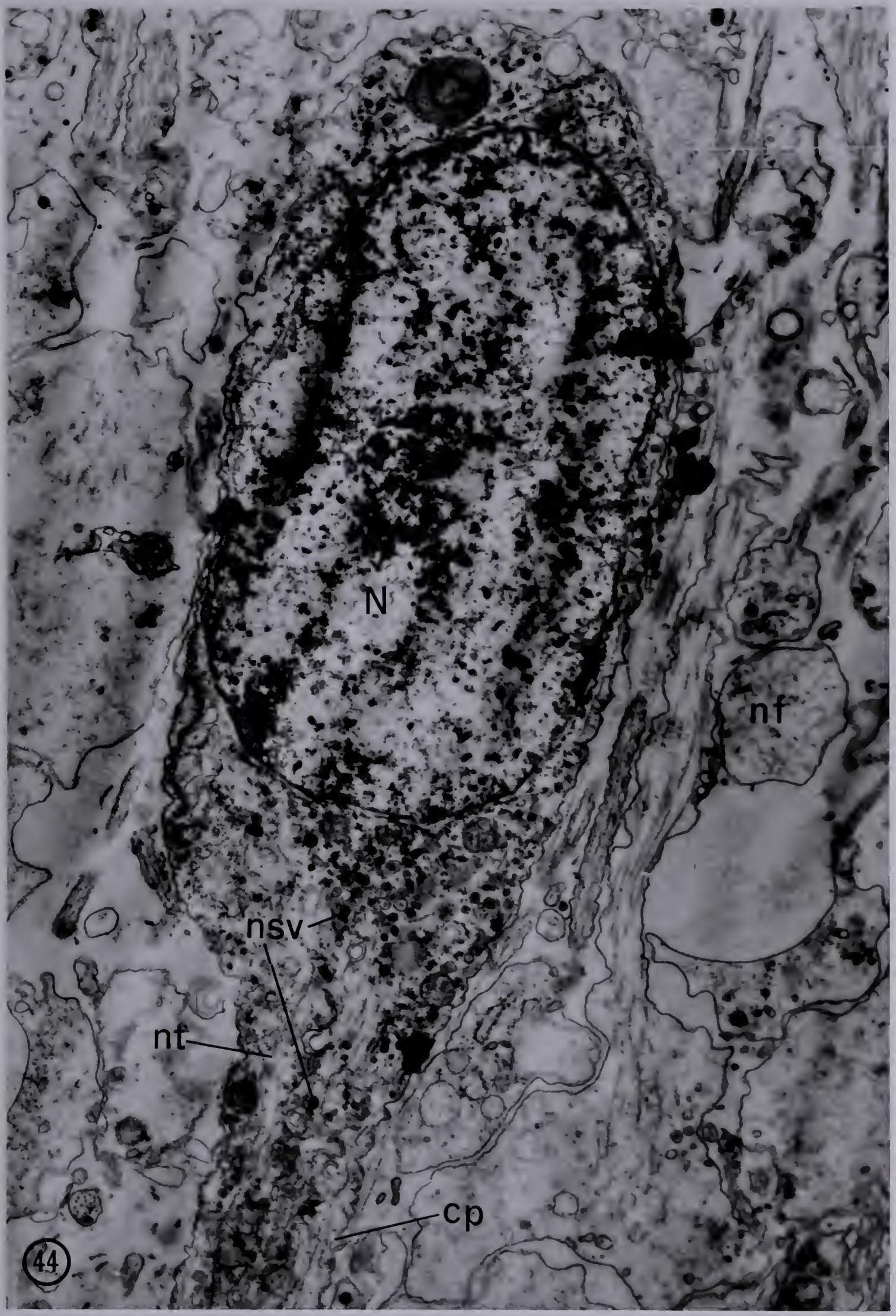

N
nf
nsv
nt
cp
44

Fig. 45. A section of the brachial nerve cord taken from a crinoid carrying ripe pinnules. Electron micrograph shows numerous neurosecretory vesicles (nsv) present in the nerve fibres. The variation in the appearance of the vesicles from one containing a small core of dense material to one where the dense contents obscures the limiting membrane is suggested to represent a stage in the neurosecretory material.

Glutaraldehyde/osmium fixation, Epon embedding and uranyl acetate-lead citrate staining. X 14,000.

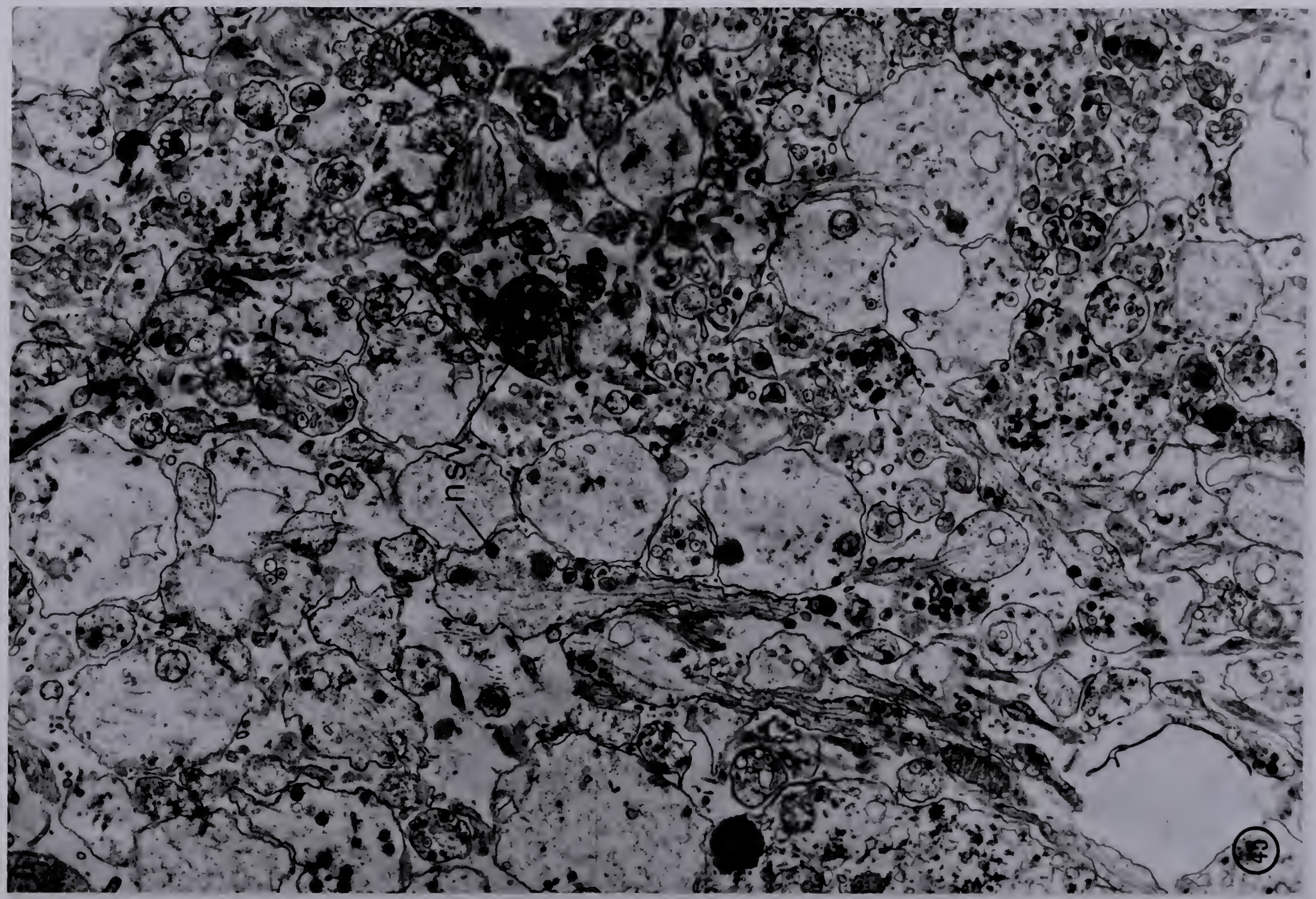

sus
n

Fig. 46. A section of a gonadal pinnule showing nerve fibres (nf) between the collagen fibres (cn) of the pinnules. Neurosecretory vesicles (nsv) are present throughout the nerves innervating the gonadal pinnules. Note the larger, more darkly staining vesicles in the cell process adjacent to the nerve fibres. These vesicles possibly contain lipid and are not related to the neurosecretory vesicles.

Glutaraldehyde/osmium fixation, Epon embedding and uranyl acetate-lead citrate staining. X 8,400.

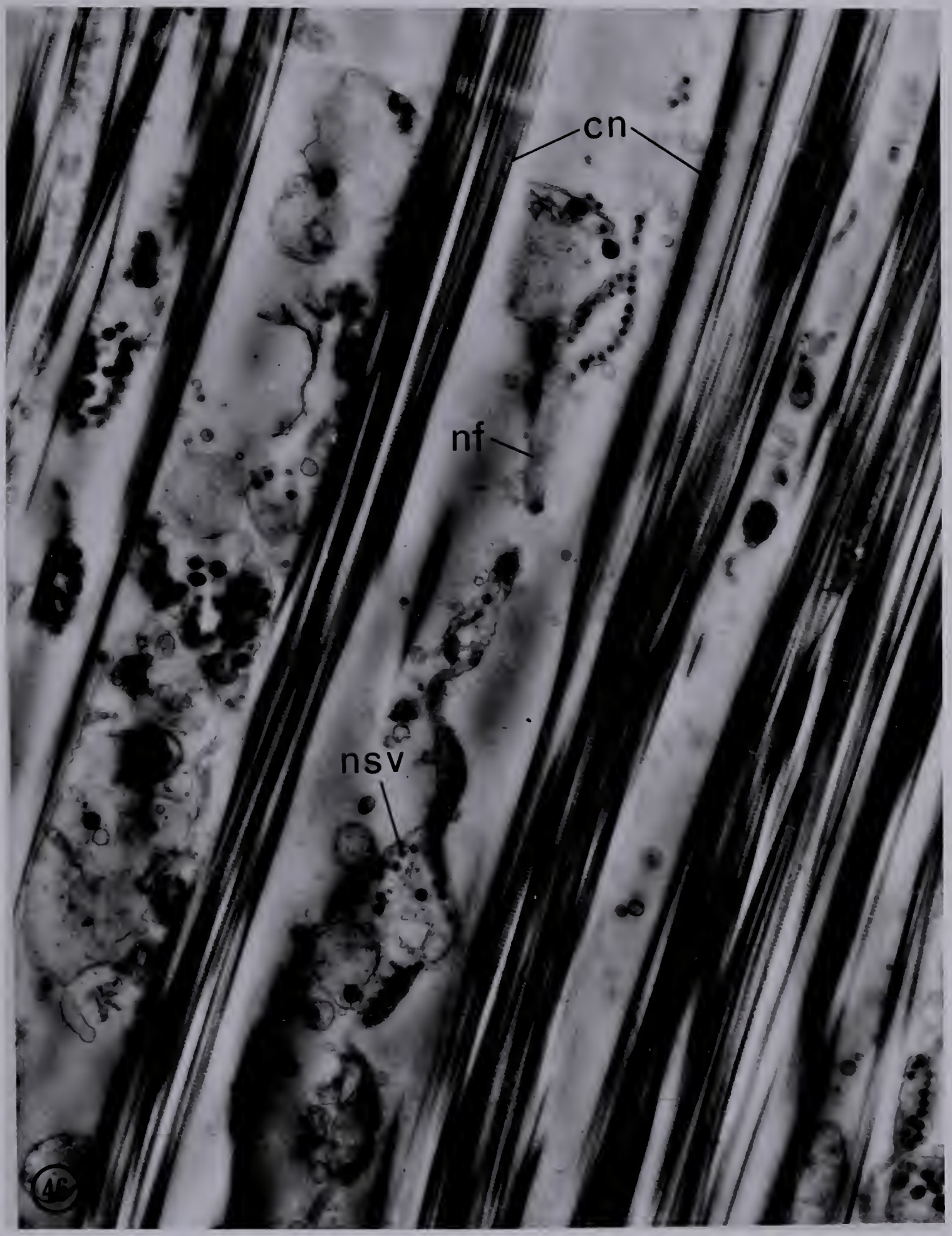
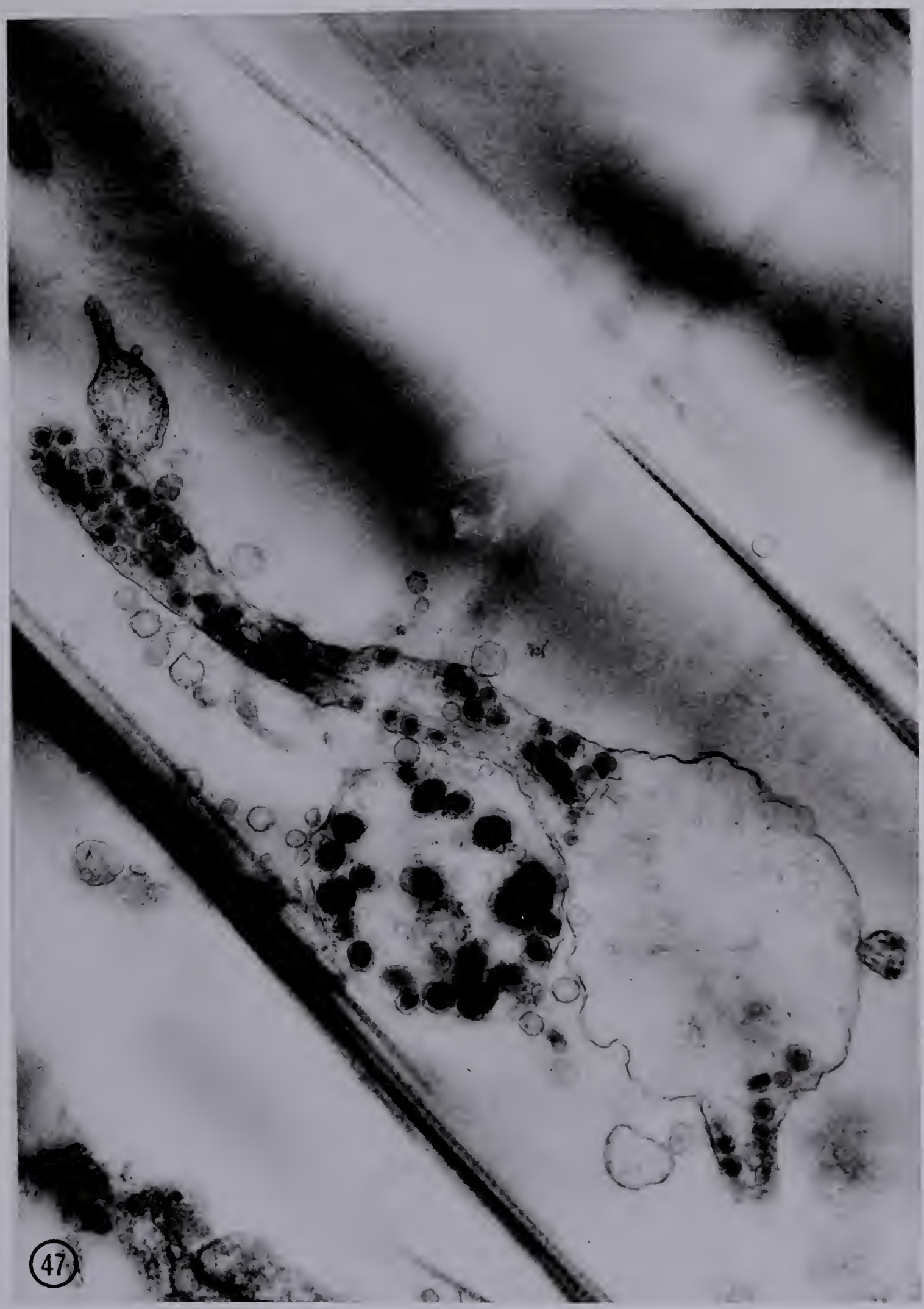

cn
nf
nsv

Fig. 47. A higher magnification of the nerve fibres between the collagen fibres between the collagen fibres of the gonadal pinnules.

Glutaraldehyde/osmium fixation, Epon embedding and uranyl acetate-lead citrate staining. X 24,000.



47